cis- and *trans*-acting Transcriptional Activators: Characterization of Single Nucleotide Polymorphisms and a Novel Two-component System of *Staphylococcus aureus*

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA

BY

Jeffrey William Hall

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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{March 2014}

Acknowledgements

First and foremost, I want to thank my advisor, Dr. Yinduo Ji, for the opportunity to work in his laboratory and being an exceptional mentor. Thank you for giving me the scientific freedom to explore my hypotheses and most importantly, fail. It may sound odd, but graduate school and science have their share of failures and I hadn't ever failed in anything I had done before graduate school. You let me explore new concepts and ideas, and if they failed, you were there to provide advice and guide me back to the correct course when I strayed. Thank you for your genuine friendship and unwavering support throughout my graduate career.

I would like to thank the other members of the Ji lab, Dr. Ting Lei, and Mrs. Junshu Yang for their support and help. Ting, you were always willing to help me and provide technical advice and I very much appreciate that. I enjoyed getting to know you over the years and I wish you great success in your future life and career. Junshu, I can't thank you enough for your hard work and help on my projects. You helped accelerate the completion of my Ph.D. work and for that, I am very thankful. I would also like to thank Drs. Chuanxin Yu and Meiying Yan for their work on the *yhcSR* TCS and creating useful plasmids and microarray data that was very useful in my thesis experiments.

Funding for this work was provided by the National Institutes of Health (AI057451), MAES Fund, Animal Hatch Fund, Network on Antimicrobial Resistance in *Staphylococcus aureus*, and the United States Department of Agriculture (AES0063105 and AES0063082.)

I would also like to thank my Ph.D. committee members, Dr. Sandy Armstrong, Dr. Gary Dunny, and Dr. Richard Isaacson for taking the time to serve as my defense committee and their constructive feedback over the years. Thank you for the support and encouragement you have provided me throughout this process.

Dr. Juan Abrahante and Bev Bybee, thank you for being wonderful people. Juanito, you have a great sense of humor and such a positive attitude, don't ever lose it. If I was disappointed in my research, looking to laugh, or needed advice, you were there to help. Thanks. I'll see you on the Dark Side.

Bev, thank you for always being willing to help me and provide any reagents that you may have had that I was looking for. I always looked forward to running into you in the media preparation room and discussing everyday topics or the last loss by the Twins or the Vikings. Hopefully, someday soon they'll stop disappointing us.

I have met many wonderful people during my time as MICaB student, and I cherish the fun times and good memories we made together, some of which we may not always remember. A special thanks to Brandon Leonard, Ryan Flynn and their respective significant others, Bobbi Leal and Megan Mutlhaup. You are a wonderful and supportive group of friends and I hope you will always remain a part of my life.

Many kids are told by their family they should go to college, but I'm not sure how many have explicit memories at the age of 8 years old sitting around their grandparent's dining table with everyone telling them they should study a

lot, work hard, and go to college, but these are memories I have and I must thank my family for that. Those memories were, and continue to be, used as motivation to complete graduate school and work hard to develop a meaningful scientific career. To my parents, thank you for your unconditional love and support.

Lastly, but certainly not least, my fiancée, Valerie. Thank you for taking a chance and moving halfway across the country with me. You are a constant source of encouragement, love, and a pillar of strength, one that I have had to lean on many times and you have never wavered. I can't thank you enough for that. I am indebted to you and will be by your side in support of your future endeavors.

Dedication

This dissertation is dedicated to my family from whom I learned that success does not come easy and that a person must work hard, have patience, and never give up on completing goals. It is also dedicated to my beautiful future-wife, Valerie, for her love and support. Thank you.

Abstract

Staphylococcus aureus is a major opportunistic pathogen and a common cause of hospital- and community-acquired infections. Furthermore, infections of livestock animals by *S. aureus* results in billion dollar losses to agriculture producers annually. Over the last five decades antibiotic resistance has dramatically increased in *S. aureus* and highly pathogenic strains have emerged that threaten human and animal health. Characterization of highly pathogenic strains and novel transcriptional mechanisms and pathways is of utmost importance as it will provide a critical evolutionary understanding of the transcriptional changes that led to the emergence of successfully infectious *S. aureus* strains and may identify novel targets for antibacterial development.

The overarching goal of research described in this thesis was to characterize and understand how novel *cis*- and *trans*-acting factors affect gene expression in *S. aureus*. To that end, the work and data presented investigate the effect of promoter based single nucleotide polymorphisms (SNPs) of the *hla* gene, encoding α-toxin, on gene transcription and gene product expression. The *cis*-acting SNPs increased the binding affinity of the promoter to the *trans*-acting transcription factor SarZ. Furthermore, the *S. aureus* RF122 strain had increased transcriptional expression of several positive regulators and decreased transcription of negative regulators of *hla*, which resulted in a dramatic increase in α-toxin expression and likely contributes to the increased mastitis pathogenesis of RF122.

Additionally, the essentiality of the *yhcSR* two-component system was confirmed in the hospital-acquired methicillin resistant *S. aureus* WCUH29 strain. The YhcSR TCS was identified to transcriptionally activate the *lacABCDE* and *opuCABC* operons involved in cellular metabolism and osmoregulatory mechanisms, respectively. In an effort determine if a relationship existed between YhcSR and pathogenesis, studies revealed that the YhcSR TCS transcriptionally regulated, in a positive manner, the *sspABC* and *crtOPQMN* operons, encoding exported proteases and staphyloxanthin biosynthesis, which contribute to the survival of *S. aureus* in human blood. The data indicate that the YhcSR TCS system is an essential *trans*-acting global regulator in *S. aureus*.

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Licensed content author	Jeffrey W. Hall,Yinduo Ji
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Chapter 1 : Introduction

1.1 Infections

In 1881, Sir Alexander Ogston reported the observation of micrococci in the pus of an infected surgical site on a knee while investigating the cause of blood poisoning (1). We now know the organism described was *Staphylococcus aureus*, a commensal and opportunistic pathogen. *S. aureus* infects tens of thousands of people annually in the US (2, 3). Methicillin-resistant *S. aureus* (MRSA) accounts for more than 60% of all hospital-acquired *S. aureus* infections (4–6). MRSA has increasingly spread beyond healthcare facilities and emerged as a community-acquired (CA) pathogen causing minor skin and soft tissue infections and severe invasive diseases, including necrotizing pneumonia and a sepsis syndrome (2, 3, 7). In the US, treating MRSA infections in hospitalized patients costs an estimated $3.2 - $4.2 billion annually and is estimated to be the causative agent of an estimated 18,650 American deaths annually (7). *S. aureus* produces an array of innate immune inhibiting factors to promote its own survival within a host (8), many of which are controlled by central key transcriptional regulatory systems (9–12). Understanding the regulatory network of *S. aureus* is important to our understanding of how *S. aureus* initiates and maintains an active infection. This knowledge will provide new insights staphylococcal infection and may provide novel targets for innovative treatments against this persistent threat to human health.

1.2 The bacterium

Staphylococcus aureus is a member of the low G+C Gram-positive firmicute phylum. The *S. aureus* cell is 0.5-0.6 μm in diameter and replicates by asexual binary fission, but the coccal daughter cells do not separate from each other, resulting in grape-like clusters when viewed under a microscope. The metabolism of *S. aureus* has been studied for approximately a century, beginning with the laborious task of determining the minimal components required to sustain aerobic cell growth (13). This breakthrough was followed shortly thereafter with the discovery of the minimal components for *S. aureus* anaerobic cultivation (14) and the individual systems essential for minimal medium growth have been investigated (15). *S. aureus* maintains complete Embden-Meyerhof-Parnas (glycolytic), pentose phosphate, and tricarboxylic acid (TCA) cycles, providing the bacterium the ability to synthesize all macromolecules from 13 basic biosynthetic intermediates; however, *S. aureus* often shows autotrophy for several amino acids through an as yet unknown mechanisms (16). *S. aureus* preferentially catabolizes glucose, but has catabolic genes for galactose and lactose utilization as well (17). Glycolytic and pentose phosphate cycle enzymes are expressed at high levels in the presence of excess glucose while TCA cycle enzymes are suppressed (18–21). Glycolysis produces two molecules of pyruvate for every one molecule of glucose consumed and reduces two molecules of NAD^+ to NADH, which are shuttled to the electron transport pathway for re-oxidation. Pyruvate is further oxidized to acetyl-CoA and CO_2 during aerobic growth, producing acetate and additional ATP (22, 23). Proteome

analysis suggests much of the cellular ATP is produced via substrate level phosphorylation when *S. aureus* is cultured in rich media (22, 24).

As a facultative anaerobe, *S. aureus* regenerates NAD^+ for glycolysis by dissimilatory nitrate reduction or fermenting several sugars and amino acids to maintain redox balance in the bacterial cell. For energy supplementation or in the absence of an alternative terminal electron acceptor, *S. aureus* relies on mixed-acid and butanediol fermentation to recycle NAD^+/NADH during anaerobic growth. Metabolite analysis of *S. aureus* cultured in a complex medium indicates *S. aureus* primarily consumes glucose, arginine and threonine, while ethanol, lactate and ornithine mainly accumulate as a result of fermentation. The pyruvate dehydrogenase complex and enzymes of the tricarboxylic acid cycle are down-regulated to prevent excess NADH production in the absence of oxygen (24, 25).

1.3 The niche

S. aureus is most frequently isolated from the anterior nares of the human nasal cavity, but the bacterium can be isolated from a range of areas on the skin and mucous membranes of humans and animals (26). *S. aureus* nasal carriage rates vary depending on a multitude of factors including age, gender, ethnicity and genetics, but at any point in time, *S. aureus* can be isolated from 20-30% of the human population (26–29). Isolation of *S. aureus* from the anterior nares is associated with a higher rate of subsequent isolation at other points on the body. A multi-center study found the endogenous *S. aureus* strain was identical to the isolated bacteremia causing *S. aureus* isolate in greater than 80% of patients (29). This conclusion highlights the well-documented observations that carriage

of *S. aureus* is a risk factor for subsequent nosocomial infections (30), as often the patient's first line of defense – the skin – is breached in some manner (26). The practice of screening for *S. aureus* and decolonizing, typically by application of mupirocin to the nasal cavity, if the bacterium is detected has been promoted as an effective means to reducing nosocomial infections, but the benefits and outcomes of decolonization have been mixed when scientifically controlled and studied (31–33).

1.4 Genetic diversity

As a species, *S. aureus* is classically differentiated from other closely related bacterial species by the formation of golden circular colonies and testing positive in the catalase assay. The availability of inexpensive and fast DNA sequencing analysis has led to an exorbitant amount of detailed genetic and evolutionary data published revealing immense genetic diversity within *S. aureus* (34–43). Collectively, published genomic analyses revealed the average *S. aureus* genome to be ~2.8 Mb, but the species has widespread variation among isolates, with as much as ~20% of the genetic content considered dispensable (37). This genetic variability is centered around antibiotic resistance and virulence factor acquisition, commonly encoded on mobile genetic elements and phages (35, 41). Genetic variability is strictly limited to these factors; single nucleotide polymorphisms within the core genome have been shown to have dramatic effects of virulence and gene expression (38, 44, 45). Additionally, at least two examples of a large recombination event, replacing 10% and 20% of

the *S. aureus* chromosome, have been documented, leading to pandemic clonal linages (46).

Much of the evolutionary genetic analysis for *S. aureus* has focused on emergence and development of antibiotic resistance as well as strain and clonal expansion of methicillin-resistant *S. aureus* (MRSA) strains. The genetic determinant for methicillin resistance is encoded by the *mecA* gene carried on the staphylococcal cassette chromosome (SCC*mec*). The gene product is an alternative penicillin binding protein, PBP2a. This protein has reduced affinity, relative to the endogenously encoded PBP2, for a broad spectrum of β-lactam antibiotics, allowing for continued cell-division in their presence (47–51). The origin of methicillin resistance is not presently clear. Evidence suggests the origin of *mecA* may have originated in the methicillin-susceptible *Staphylococcus sciuri*, but evidence is lacking on how this normal physiological functioning protein evolved to give rise to β-lactam resistance in *S. aureus* (52). What is clear, however, is methicillin-resistance has arisen independently multiple times in *S. aureus* and is the result of horizontal gene transfer of SCC*mec* from MRSA to methicillin-susceptible *S. aureus* progenitor strains (53).

On a broad scale, three groups of *S. aureus* lineages can be defined: livestock associated (LA-MRSA), healthcare-associated (HA-MRSA), and community-associated (CA-MRSA). The emergence of LA-MRSA is of great concern as zoonotic infections between caretakers and animals have been documented and genetic analyses of LA-MRSA isolates suggest they contribute significantly to the overall burden of MRSA infections (54). Interestingly, genetic

analysis of LA-MRSA isolates indicates the progenitor was likely a human methicillin sensitive *S. aureus* (MSSA) strain. This strain likely rapidly adapted to livestock, even losing human-specific immune modulators, but gaining resistance to methicillin and tetracycline, two antibiotics associated with feedstock (55–57).

The commercial development of penicillin as a tool to fight Gram-positive bacterial infections was dramatic and immediate, but the overuse and misuse of the drug was its downfall. Within four years of the antibiotic's introduction, penicillin-resistant infections were reported (26, 58). The introduction of methicillin to treat penicillin-resistant *S. aureus* infections was short lived as well. Just two years after its licensing for use in England, the first isolate of MRSA was reported in 1961. By 1968, MRSA had been reported in the United States (39). Since then a common theme has emerged; a new antibiotic is introduced, and within a short period of time, resistant isolates arise. The genome of *S. aureus* is highly plastic, but conjugation and transduction are not solely responsible for the development of drug-resistant strains. *S. aureus* is also capable of developing resistance during the course of an infection, resulting from point mutations in the core genome (43). HA-MRSA infections often form at the site of surgical incisions or repeated skin breaks (often occurring in diabetics or dialysis patients) due the repeated administration of antibiotics over a long period of time (7, 59).

In the last two decades, MRSA has moved from being a strictly hospital-associated infection to an infection regularly acquired in community settings. These CA-MRSA strains were initially thought to be the result of "escaping" HA-MRSA strains, but genetic analyses of these isolates revealed they have minimal

genetic relatedness to their HA-MRSA counterparts. Most notably, CA-MRSA strains carry smaller, distinct SSC*mec* elements and are often Panton-Valentine Leukocidin (PVL) positive (60). The mere presence of PVL does not explain the global expansion of CA-MRSA and is likely related to the diverse genetic backgrounds and antibiotic susceptibilities of CA-MRSA strains (61). It has been suggested the emergence of CA-MRSA has been a complex and progressive evolutionary development of distinct non HA-MSSA strains which gained virulence factors and antibiotics, going unnoticed by medical professionals for many years (62). While invasive infections are possible, CA-MRSA infections more commonly result in purulent skin and soft tissue infections.

1.4.1 Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) is a variation in the DNA sequence at the same site between two isolates of the same biological species. Synonymous SNPs are silent and have a minimal role in the evolution of the organism, as they do not alter the expression level or primary protein sequence; however, SNPs may be useful for genetic typing of the species and evolutionary analyses (63). Non-synonymous SNPs may alter the expression level of genes if they occur in regulatory regions (called eSNPs). Alternatively, if non-synonymous SNPs occur in the coding region of a gene, they may result in missense or nonsense mutations.

Identifying and classifying isolates of *S. aureus* are common laboratory practices and are important epidemiologically to determine colonality and prevalence of isolates, as well as follow outbreaks (64). Several molecular techniques utilize

SNPs to group isolates of *S. aureus*: multi-locus sequence typing; pulsed field gel electrophoresis; and PCR coupled with sequencing-based assays, such as SCC*mec*, *spa*, *coa* and *agr* typing (65–70). Additionally, genetic analysis has shown SNPs have a prominent role in the development of antibiotic resistance (71–73). For example, SNPs within the *mprF* gene and *rpoB* result in resistance to daptomycin and rifampicin, respectively (74, 75). On a large scale, SNPs are useful for high-resolution epidemiological surveillance of *S. aureus* outbreaks (76–78).

Bacterial genomes are not static and whole-genome sequencing has revealed SNPs to be the largest source of genetic diversity with a bacterial species. Additionally, SNPs are often the result of individual environmental experiences exerting positive selection forces on individual genomes that lead to adaptive changes (79–81). SNPs within promoter regions can affect virulence factor expression (82) and may even dictate host specificity (83). For example, an SNP present within the promoter region of the thermostable direct haemolysin gene of *Vibrio parahaemolyticus* up-regulates haemolysin expression (84). SNPs contribute to the strain-specific characteristics of *S. aureus* and other bacteria, acting as endogenous sources of experimental genetic variation with the end result to enhance fitness (77, 85, 86).

The rate and level of transcription of a gene is affected by several factors, including the promoter affinity for DNA-dependent RNA polymerase and the presence of transcription factors. Bacteria possess only one RNA polymerase (RNAP) which is responsible for all transcription within the cell. An RNAP is capable of initiating transcription by itself, but must be associated with a sigma factor, which is responsible

for DNA sequence recognition, to create a holo-RNAP capable of extended transcription (87). An archetypal bacterial promoter consists of a pair of hexanucleotide consensus motifs, termed the -10 (TTGACA) and -35 boxes (TATAAT), separated by a ~17 bp spacer and is recognized by the housekeeping sigma factor, termed σ^A in Gram-positive bacteria (88). This archetypal promoter is the most efficient promoter of gene transcription, but SNPs within the consensus motifs alter RNAP's affinity for the promoter, reducing the rate of transcription, providing the first level of transcriptional regulation (89).

DNA-binding transcription factors (TF) are proteins which regulate the expression of genes in response to an internal or external stimuli. They bind *cis* acting TF binding sites and, much like RNAP and sigma factors, SNPs within the *cis* acting elements alter the binding site from the optimal consensus sequence of the respective TF and dictate the affinity of the TF for DNA. The SNPs subsequently alter the efficiency of the TF to regulate transcription via interaction with RNAP (90).

In experiments described in chapter two, the differential expression *hla*, encoding α-toxin, between bovine and human isolates of *S. aureus* is linked to the presence of SNPs in the *hla* promoter region that increase the DNA-binding affinity of the activating SarZ TF, leading to hyperproduction of α-toxin.

1.5 Two-component signal transduction systems

S. aureus possesses many genes that allow the organism to sense and adapt to ever-changing environmental conditions. At least 16 operons encode two-component signal transduction systems (TCSs) in *S. aureus* (17), with 14 conserved in all sequenced genomes (91). These TCSs sense environmental

conditions and manipulate cellular responses accordingly. Cellular responses frequently result in increased resistance to antibiotics (73, 92); regulated expression of accessory genes critical for infection (45, 93–97); and necessary metabolic changes (98–101).

A prototypical TCS consists of a dimerized sensor histidine kinase (HK) which responds to a specific environmental cue(s) by transautophosphorylating and subsequently transferring the phosphate to a cognate response regulator. Phosphorylation of the response regulator activates the effector function of the protein. The effector function ultimately leads to modulation of gene expression within the TCS regulon, mediating the cellular response to the environmental cue (102–105). A detailed structural analysis of the many subclasses of TCSs can be reviewed in Mascher et al. (106) and M.Y. Galperin (107).

At least 16 operons encode two-component signal transduction systems (TCSs) in *S. aureus* (17), with 14 conserved in all sequenced genomes (91), which sense environmental conditions and manipulate cellular responses accordingly. Many TCSs have been investigated and their roles within the *S. aureus* are well understood as identified in Table 1-1. Typically the loss of a TCS is not lethal to the bacterium under *in vitro* growth conditions, but it may alter the normal gene expression and/or phenotype of the cell. There are two known exceptions to the non-essentiality of *S. aureus* TCSs, the *walKR* (*vicRS, yycGF*) TCS and *yhcSR* TCS, which have been shown to be indispensable for bacterial cell viability. The *walKR* system is a major regulator of cell wall metabolism within the *S. aureus* cell (108). Independently, the genes controlled by WalKR are not

essential (109, 110); WalKR positively regulates at least five major genes involved in cell wall metabolism and the deletion of *walKR* likely leads to a synergetic loss of cell wall metabolism enzymes that results in cell death (108). Recently, novel inhibitors have been identified for the WalKR system and continued research will determine if these chemicals will be therapeutically beneficial (111–115).

Table 1-1 Two-component systems of *Staphylococcus aureus*

TCS	Gene(s) or locus names[a]	Function	Reference(s)
1	*agrCA*	quorum sensing and accessory gene regulation	(116)
2	*saeRS*	exo-protein regulation and innate immune evasion	(45, 117, 118)
3	*arlRS*	virulence and biofilm regulation	(119, 120)
4	*graRS*	vancomycin resistance and SOS response	(92, 121)
5	*vraRS*	vancomycin resistance and peptidoglycan remodeling enzymes	(122)
6	*lytRS*	programmed cell death and autolysis	(123, 124)
7	*ssrAB (shrAB)*	anaerobic growth and TSST-1 regulation	(125, 126)
8	*nreBC*	dissimilatory nitrate reduction regulation	(101, 127)
9	*walKR (yycFG)*	cell wall metabolism regulation; essential	(108)
10	*yhcSR (airSR)*	aerobic/anaerobic growth and exported protein regulation; essential	(100, 128–130)
11	*kdpDE*	extracellular K+ sensing	(98)
12	*hhsRS*	heme sensing and detoxification	(131)
13	*braSR*	bacitracin resistance associated	(132)
14	SA0216-SA2015	unknown	
15	SA1158-SA1159	unknown	
16	SA1516-SA1515	unknown; *phoP/phoR*-like	

[a] Loci numbers based on *Staphylococcus aureus* N315 sequence (17)

The majority of response regulators are helix-turn-helix DNA-binding transcription factors and their effector function is transcriptional regulation (102, 107). In cases of transcriptional activation the response regulator binds an upstream binding site and facilitates loading of the holo-RNAP on the promoter via interaction with the alpha C-terminal domain of the RNAP complex. Alternatively, the response regulator binding site may overlap the -35 motif, in which case the response regulator directly binds the sigma factor in the holo-RNAP facilitating transcription initiation (87). AgrA binding to the region between the P2 and P3 of the divergent RNAII and RNAIII promoters is an example of a third mechanism of positive regulation, whereby response regulator binding induces a conformational change in the DNA and increases access to the promoter so the holo-RNAP can bind the promoter elements (133). In studies described in chapters 4-6, positive transcriptional regulation of the *lacABCDE, opuCABCD, sspABC,* and *crtOPQMN* by the YhcSR TCS is presented.

1.6 Thesis rationale and goals

Alpha-toxin is an important virulence factor for many types of *S. aureus* infections in humans and animals (134, 135). Infections by *S. aureus* impose enormous financial burdens on human public health services and animal agriculture producers. The emergence of a hyper-producing alpha-toxin sub-type of *S. aureus* poses a public health risk and impacts the financial interests of animal producers. The goal of chapter 2 was to identify the mechanism(s) that are responsible for the up-regulation of alpha-toxin in the *S. aureus* RF122 strain.

Two-component signal transduction systems are the second largest family of transcription factors in *S. aureus* (91), with prominent roles in metabolism, cellular division, and virulence factor production. These systems are a critical part of the intricate genetic regulatory system in *S. aureus*. While most of the TCSs have been studied, some extensively, a handful remain to be characterized. One of these, the *yhcSR* TCS, is of special interest because it was found to be essential in the *S. aureus* RN4220 laboratory strain, making it a potential new target for novel antibiotic development. The goal of this section of the dissertation is to verify the essentiality of *yhcSR* in clinical isolates of MRSA, identify the underlying mechanism of essentiality through characterization of inducible mutants and identification of regulated genes, and determine if *yhcSR* had a role in pathogenesis.

1.7 Summary of thesis

The underlying mechanisms of hyper-production of alpha-toxin (*hla)* in *S. aureus* RF122 are three-fold. At least four known positive transcriptional regulators - *sarZ, arlS, saeS,* and *agrA* - and one negative transcriptional regulator, *rot*, are up-regulated and down-regulated, respectively, compared to the low alpha-toxin producing strain, WCUH29. Additionally, the positive regulator SarZ has a higher binding affinity for the RF122 *hla* promoter, which may be related to the presence of SNPs located in the upstream promoter region of all alpha-toxin hyper-producing strains isolated in this study. The differential expression of *trans* transcriptional factors and the presence of *cis* transcriptional elements that promote up-regulation cumulatively result in an eight-fold increase

in *hla* transcription relative the low alpha-toxin expressing *S. aureus* WCUH29 strain ultimately leading to a dramatic increase of the effector toxin, alpha-toxin in *S. aureus* RF122.

As a continuation of work published in 2005 (128), the essentiality of the *yhcSR* TCS was confirmed in the HA-MRSA strain WCUH29 and *yhcS* antisense RNA was shown to inhibit growth of the CA-MRSA strain 923 and MSSA strain Newman, suggesting it is essential in those strains as well. In collaboration with a former post-doctoral associate, YhcSR was found to promote the transcription of the *lacABCDE* and *opuCABC* operons involved in lactose catabolism and cellular osmotic maintenance. Heterologous expression of these genes during depletion of *yhcSR* by antisense RNA could partially restore growth to *S. aureus*, suggesting they may be linked to *yhcSR* essentiality. As neither operon contains genes which were essential individually, it is possible the essential nature of *yhcSR* is linked to its global regulatory role. My work, and that of others (136, 137), indicate the YhcSR TCS is involved in regulation of a variety of cellular pathways and it is possible that the cumulative dysregulation of all these pathways during depletion of *yhcSR* results in cell death.

Lastly, I show the YhcSR TCS system transcriptional promotes expression of two virulence factor operons involved in innate immune system evasion. The first is the protease operon, *sspABC*. The proteases encoded by this operon SspA (V8 Protease) and SspB (Staphopain B) perform a variety of functions such as cleaving Protein A from the bacterial cell-wall (138) to proteolytically cleaving and inactivating complement factors (139) and antibody (140) to suppress innate

immune system recognition of *S. aureus.* The overproduction of YhcR promotes survival of *S. aureus* WCUH29 in heparinized human whole blood via up-regulation of the SspAB proteases which degrade opsonins and prevent phagocytosis. The second virulence factor positively regulated by YhcSR is staphyloxanthin, a golden carotenoid, produced by the *crtOPQMN* biosynthetic operon. Staphyloxanthin is important for protection against reactive oxygen species produced by phagocytic cells. The overproduction of YhcR increased staphyloxanthin production resulting in increased tolerance to hydrogen peroxide and enhanced *S. aureus* survival in whole blood. Elimination of staphyloxanthin production in the background of YhcR overproduction completely eliminated enhanced survival of *S. aureus* in human blood. Taken together, YhcSR transcriptionally regulates two virulence factor operons involved in anti-phagocytosis and immune evasion, directly linking this novel essential TCS to the pathogenesis of *S. aureus*.

Identification of Single Nucleotide Polymorphisms Associated with Hyperproduction of Alpha-toxin in *Staphylococcus aureus*

Liang X. §, Hall J.W. §, Yang J., Yan M., Doll K., et al. (2011) Identification of Single Nucleotide Polymorphisms Associated with Hyperproduction of Alpha-Toxin in *Staphylococcus aureus*. PLoS ONE 6(4): e18428. doi:10.1371/journal.pone.0018428

§ These authors contributed equally to this work.

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Chapter 2 : Identification of Single Nucleotide Polymorphisms Associated with Hyperproduction of Alpha-toxin in *Staphylococcus aureus*

2.1 Overview

The virulence factor α-toxin (*hla*) is needed by *Staphylococcus aureus* in order to cause infections in both animals and humans. Although the complicated regulation of *hla* expression has been well studied in human *S. aureus* isolates, the mechanisms of *hla* regulation in bovine *S. aureus* isolates remain undefined. In this study, we found that many bovine *S. aureus* isolates, including the RF122 strain, generate dramatic amounts of α-toxin *in vitro* compared with human clinical *S. aureus* isolates, including MRSA WCUH29 and MRSA USA300. To elucidate potential regulatory mechanisms, we analyzed the *hla* promoter regions and identified predominant single nucleotide polymorphisms (SNPs) at positions -376, -483, and -484 from the start codon in α-toxin hyper-producing isolates. Using site-directed mutagenesis and *hla* promoter-*gfp*-*luxABCDE* dual reporter approaches, we demonstrated that the SNPs contribute to the differential control of *hla* expression among bovine and human *S. aureus* isolates. Using a DNA affinity assay, gel-shift assays and a null mutant, we identified and revealed that an *hla* positive regulator, SarZ, contributes to the involvement of the SNPs in mediating *hla* expression. In addition, we found that the bovine *S. aureus* isolate RF122 exhibits higher transcription levels of *hla* positive regulators, including *agrA*, *saeR*, *arlR* and *sarZ*, but a lower expression level of *hla* repressor *rot* compared to the human *S. aureus* isolate WCUH29. Our results indicate α-toxin hyperproduction in bovine *S. aureus* is a multifactorial process, influenced at both the genomic and transcriptional levels. Moreover, the identification of

predominant SNPs in the *hla* promoter region may provide a novel method for genotyping the *S. aureus* isolates.

2.2 Introduction

S. aureus is an important pathogen capable of causing both animal and human infections such as pneumonia, endocarditis, toxic shock syndrome and bovine mastitis. The continuous increase of infections associated with both hospital- and community-acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA and CA-MRSA) has caused serious public health concerns (141). The ability of this organism to cause a wide variety of infections partially depends on the coordinated and regulated expression of multiple cell- and surface-associated virulence factors (142, 143) and exported proteins including various proteases and toxins (144).

Alpha-toxin plays a critical role in the modulation of *S. aureus*–induced cytotoxicity in Jurkat T-lymphocytes, human peripheral blood lymphocytes, monocytes (145) and epithelial cells (146), even though multiple virulence factors are required for the bacterium to induce apoptosis in endothelial cells (147). Alpha-toxin can interact specifically with surface receptors of the host cells, form functional transmembrane pores, and selectively release ions, and/or leads to the activation of cell signaling pathways, thus inducing apoptosis and/or necrosis in various cell types (148–152). Recently, we demonstrated that α-toxin can interact with α5β1-integrin to interfere with *S. aureus* adhering to and internalizing into human lung epithelial cells (A549) (153). The interaction of α-toxin with α5β1-

integrin contributes to the cytotoxicity of α-toxin that is required for *S. aureus* to induce apoptosis and death of the epithelial cells (146). However, the role played by α-toxin depends on the stage and/or type of infection and the quantities produced. It has been demonstrated that α-toxin is an important virulence factor in experimental brain abscesses and pneumonia (154–156) and intraperitoneal infection (157, 158), whereas the overproduction of α-toxin significantly reduces virulence in experimental endocarditis (159).

Alpha-toxin expression is up-regulated in the stationary growth phase *in vitro* (116) and at later stages of animal infection (160, 161). The expression of α-toxin is simultaneously regulated by different regulators, which have been well-documented and reviewed elsewhere (116, 162). The expression of α-toxin is positively regulated by various global regulators, including two-component signal transduction systems such as the accessory gene regulator (Agr) (116, 163), the staphylococcal accessory protein effector (SaeRS) (117, 164), ArlRS (165, 166) and transcriptional regulators Mgr (167) and SarZ (168–170). In contrast, the homologues of staphylococcal accessory regulator (SarA), including Rot and SarT, repress the expression of α-toxin (171, 172). The role of SarA in modulating *hla* transcription is controversial: SarA affects *hla* expression in both an *agr*-dependent and *agr*-independent manner (161, 173). In addition, it has been revealed that *hla* transcription is affected by additional factors, including the alternative sigma B factor (σ^B) and environmental stimuli (174, 175).

Alpha-toxin also serves as a virulence factor in *S. aureus*-induced mastitis (176, 177). It has been found that significant increases in milk antibodies to α-

and β-toxins are present in cows with chronic staphylococcal mastitis (178), and that many *S. aureus* isolates from the mammary gland of dairy cows produce α-toxin (179). The comparative genomic analysis of human and bovine *S. aureus* isolates suggests that a unique mechanism may be involved in the pathogenicity of bovine *S. aureus* isolates (152) and that some bovine *S. aureus* isolates generate dramatic amounts of α-toxin (135). However, the mechanism of hyperproduction of α-toxin in some bovine *S. aureus* isolates remains undefined.

In the present study, we compared the expression profiles of exported proteins between bovine *S. aureus* isolates and human clinical *S. aureus* isolates. We found hyperproduction of α-toxin in many bovine *S. aureus* isolates and investigated potential mechanisms of up-regulation of *hla* using site-direct mutagenesis, transcriptional promoter-reporter fusion, and quantitative RT-PCR approaches. Our results indicate α-toxin hyperproduction in bovine *S. aureus* is a multifactorial process, influenced at both the genomic and transcriptional levels.

2.3 Materials and methods

2.3.1 Bacterial strains, plasmids and growth media.

The bacterial strains and plasmids used in this study are listed in Table 2-1. The human *S. aureus* strains were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* or references indicated. The bovine *S. aureus* isolates were obtained from geographically diverse animals that received care at the University of Minnesota's Veterinary Diagnostics Laboratory. The *S. aureus* cells were cultured in Trypticase soy broth (TSB) at 37˚C with shaking. *E. coli* strains were grown in Luria-Bertani (LB) medium. Transformants containing recombinant plasmids were selected on LB agar containing ampicillin (100 µg/ml) for *E. coli* and TSA containing chloramphenicol (10 µg/ml) for *S. aureus*.

2.3.2 SDS-PAGE analysis of exported proteins.

The supernatants were collected from the overnight cultures of *S. aureus* isolates in TSB medium by centrifugation at 3900 x g. The exported proteins were precipitated from an equal volume of supernatant using ethanol as described (157). The exported protein profiles were detected by a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining.

Statistical analysis. Data are the means ± standard errors of the means from three experiments. The symbol "*" indicates a significant difference ($P \leq 0.05$) using an unpaired *t* test.

2.3.3 Construction and detection of promoter-*gfp-lux* dual reporter fusions.

In order to further confirm the transcriptional regulation of *hla* expression, we created *hla* promoter-*gfp*-*lux* dual reporter constructs as previously described (117). The *gfp*-*lux* dual reporter fusion system was provided courtesy of Philip Hill (180). An approximate 1kb upstream region of *hla* was amplified from both WCUH29 chromosomal DNA and RF122 chromosomal DNA by PCR, respectively, using the primers listed in Table 2-2, digested with *EcoR*I and *Xma*I, and ligated into the upstream region of the promoterless *gfp*-*lux* of pCY1006, which was digested with the same enzymes. The resulting recombinant plasmids pXL1107 and pXL1207 were electroporated into *S. aureus* RN4220, then into both WCUH29 and RF122, resulting in *S. aureus* strains SaRN1107, SaRN1207, SaWH1107, SaWH1207, SaRF1107 and SaRF1207. The *lux* expression was monitored until early stationary phase in TSB with an appropriate antibiotic at 37°C with a Chiron luminometer. The relative light units (RLU) were calculated (bioluminescence intensity/optical density at 600nm). For western blot analysis of Gfp expression in different *hla* promoter-*gfp* fusions in RF122, a 1:100 dilution of an overnight culture was grown and the same number of bacterial cells was harvested from cultures at an optical density of 1 at 600 nm by centrifugation. The whole cell lysates were prepared, and the same volume of lysate was loaded on 12% SDS-PAGE and probed by rabbit anti-Gfp antiserum using western blot assay. The density of the reaction band in equal area was scanned and calculated using ImageJ software.

2.3.4 Site-directed mutagenesis.

To determine whether *hla* transcription is modulated by single nucleotide polymorphisms (SNPs) in the RF122 *hla* promoter, site-directed mutations were generated by PCR using the pXL1207 as a template and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions except that primer extension was allowed to continue for 8 min. The primers used for site-directed mutagenesis are listed in Table 2-2. The mutations were confirmed by DNA sequencing of the region containing the mutation. The reformed plasmids were designated pXL1307, pXL1407 and pXL1507, and electroporated into RN4220, then into WCUH29 and RF122. The resulting *S. aureus* strains were named SaRN1307, SaRN1407, SaRN1507, SaWH1307, SaWH1407, SaWH1507, SaRF1307, SaRF1407 and SaRF1507.

The chromosomal DNA from each bovine mastitis *S. aureus* isolate listed in Table 2-1 was purified and the promoter region of *hla* from each isolate was obtained by PCR using the same primer listed in Table 2-2. The PCR products were purified and sequenced; the DNA sequences were deposited in GenBank (accession #HQ592340 to HQ592346).

2.3.5 DNA affinity purification of *hla* promoter region binding proteins.

In order to identify regulators associated with SNPs, we utilized Dynabeads M-280 strepavidin coated paramagnetic beads (Invitrogen, Carlsbad, CA) to identify DNA-binding proteins according to the manufacturer’s protocol. A 265 bp PCR fragment spanning the RF122 *hla* promoter SNP region was PCR amplified using the primers RF122-phla217for and phlarev482 listed in Table 2-2

and purified using a PCR cleanup kit (Promega, Madison, WI). Approximately 9 µg of purified biotinylated *hla* promoter region was mixed with the beads. The mixtures were incubated at room temperature for 30 minutes with occasional gentle mixing. The beads were washed and resuspended in Protein Binding Buffer (10 mM Tris–HCl pH 7.5, 50 mM NaCl, and 1 mM DTT), then mixed with 500 µg of total cytoplasmic protein of RF122. The reaction mixtures were incubated at room temperature for 30 minutes with gentle, occasional swirling. The extra protein supernatant was removed and the beads were washed with Protein Binding Buffer to remove nonspecific binding proteins before being resuspended in 28 µl of Elution Buffer (10 mM Tris–HCl pH 7.5, 10% glycerol, 1 M NaCl, and 1 mM DTT) and incubated at room temperature for 30 minutes with occasional vortexing. A portion of the cytoplasmic protein fraction, washes, and elutes from the beads were detect by SDS-PAGE and visualized by Coomassie Blue staining.

2.3.6 Construction of *sarZ* null mutant in RF122 strain.

Construction of *sarZ* null mutant in RF122 isolate was performed by phage transduction as described (170). Dr. Adhar Manna provided us with a *sarZ* mutant of RN6390 (AM1090). A phage Φ80α lysate of AM1090 was prepared to infect bovine *S. aureus* isolate RF122 to create a *sarZ* null mutant of RF122, which was confirmed by diagnostic PCR and DNA sequencing of the flanking regions of *sarZ*. This null mutant was designated as BSasarZ. The different *hla*-promoter-*gfp*-*lux* reporter fusions pXL1107, pXL1207, pXL1307, pXL1407, and pXL1507, were each electroporated into BSasarZ. The resulting *S. aureus*

strains were named BSasarZ1107, BSasarZ1207, BSasarZ1307, BSasarZ1407, and BSasarZ1507.

2.3.7 Cloning, expression, and purification of recombinant SarZ protein.

The *sarZ* coding region was obtained by PCR using *sarZ* specific primers (sarZNdeIfor1 and sarZXhoIrev) listed in Table 2-2 from *S. aureus* and cloned into *Nde*I and *Xho*I sites of the *E. coli* expression vector pET24b. The recombinant DNA (pET*sarZ*) was confirmed by PCR and DNA sequencing and transformed into *E. coli* strain BL21. The transformants were incubated until mid-log phase (OD600nm = ~0.4); followed by induction of *sarZ* expression by adding IPTG (final concentration 1mM). The His-tagged SarZ protein expression and purification were conducted as described (128). The purity of purified His-tagged SarZ protein was evaluated in a 12% SDS-PAGE followed by Coomassie Blue staining.

2.3.8 Gel shift assays.

The primers (Phla217for and Phlarev428) used for gel shift analysis are listed in Table 2-2. A 265 bp DNA fragment of hla promoter upstream region encompassing the SNPs was obtained by PCR using either RF122 chromosomal DNA or WCUH29 chromosomal DNA as a template. The amplified DNA fragments were purified and labeled with Digoxigenin using the DIG GEL Shift Kits (Roche, Madison, WI) according to the manufacturer's protocol. The DNA-binding and electrophoresis were performed as described (170). Briefly, the purified PCR products were labeled with Digoxigenin using terminal transferase (Roche, Madison, WI). The interaction of SarZ with DNA was conducted in a 10µl

reaction mixture containing 0.03pmol DIG-labeled DNA, 1μg of poly-(dI–dC), 25mM NaH_2PO_4 (pH 8.0), 50mM NaCl, 2 mM $MgCl_2$, 1mM DTT, 10% glycerol and increasing amount of SarZ protein. An unlabeled DNA fragment of the promoter region as a specific competitor was added into the reaction with 100-fold excess to the labeled probe. After incubation at 25 ^oC for 20 min, the reaction mixtures were analyzed by 5% native PAGE.

2.3.9 RNA purification and quantitative RT-PCR analysis (qPCR).

Overnight cultures of *S. aureus* (WCUH29 and RF122) were incubated in TSB medium and grown to the mid-exponential phase of growth (OD_{600nm} ~0.5) at 37°C with shaking. Cells were harvested by centrifugation at 3900 x g, and the RNA was isolated by the RNAPrep Kit (Promega, Madison, WI) according to the manufacturer's instructions. Contaminating DNA was removed with the TURBO DNA-*free* Kit (Ambion, Austin, TX) and the RNA yield was determined spectrophotometrically at 260nm. The integrity of the purified RNA was analyzed by electrophoresis in 1.2% agarose-0.66 M formaldehyde gels. The 23S and 16S rRNA bands were clear without any obvious smearing patterns.

The first strand cDNA was synthesized using reverse transcriptase SuperScript III (Invitrogen, Carlsbad, CA). For each RNA sample, duplicate reactions of reverse transcription were performed in order to determine the levels of DNA contamination; there was also a control without reverse transcriptase. PCR reactions were set up in triplicate by using the SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA). Real-time sequence-specific detection and relative quantification were performed with the Stratagene Mx3000P Real Time PCR

System. Relative quantification of the product was calculated using the Comparative CT method, as described for the Stratagene Mx3000P system. The housekeeping gene 16S rRNA was used as an endogenous control (117). All samples were analyzed in triplicate and normalized against 16S rRNA gene expression.

Table 2-1 Bacterial strains and plasmids

Strain	Description	Source
RN4220	laboratory *S. aureus* strain (*rsbU*$^-$)	(181)
WCUH29	human clinical MRSA isolate	NCIMB40771
COL	human MRSA isolates	NARSA
NRS105-NRS384	human MRSA isolates	NARSA
MW2 and USA300	human clinical MRSA isolate	(182)
RF122	bovine mastitis *S. aureus* isolate	(47)
BSasarZ	*sarZ* mutant of RF122 with *sarZ*::*ermC*	This study
BSa12-Bsa110	bovine mastitis *S. aureus* isolates	CVM Dia. Lab.
SaRN1107	RN4220 carrying pXL1107	This study
SaRN1207	RN4220 carrying pXL1207	This study
SaRN1307	RN4220 carrying pXL1307	This study
SaRN1407	RN4220 carrying pXL1407	This study
SaRN1507	RN4220 carrying pXL1507	This study
SaWH1107	WCUH29 carrying pXL1107	This study
SaWH1207	WCUH29 carrying pXL1207	This study
SaWH1307	WCUH29 carrying pXL1307	This study
SaWH1407	WCUH29 carrying pXL1407	This study
SaWH1507	WCUH29 carrying pXL1507	This study
SaRF1107	RF122 carrying pXL1107	This study
SaRF1207	RF122 carrying pXL1207	This study
SaRF1307	RF122 carrying pXL1307	This study
SaRF1407	RF122 carrying pXL1407	This study
SaRF1507	RF122 carrying pXL1507	This study
BSasarZ1107	BSasarZ carrying pXL1107	This study
BSasarZ1207	BSasarZ carrying pXL1207	This study
BSasarZ1307	BSasarZ carrying pXL1307	This study
BSasarZ1407	BSasarZ carrying pXL1407	This study
BSasarZ1507	BSasarZ carrying pXL1507	This study
Plasmids	**Description**	**Source**
pCY1006	Shuttle vector carrying *agr* promoter-*gfp*-*lux* reporter, derives from pSB2019, CmR, AmpR	31
pXL1107	WCUH29 *hla* promoter-*gfp*-*lux* reporter	This study
pXL1207	RF122 *hla* promoter-*gfp*-*lux* reporter	This study
pXL1307	RF122 (T→G) *hla* promoter-*gfp*-*lux* reporter	This study
pXL1407	RF122 (TC→AT) *hla* promoter-*gfp*-*lux* reporter	This study
pXL1507	RF122 (T→G/TC→AT) *hla* promoter-*gfp*-*lux* reporter	This study

Table 2-2 Oligonucleotide sequences

Sa0660RT	For 5'-CATTGCTATTAGCGATGAAGGTATTGG-3'
	Rev 5'-CTGCTTACACTGATTTTTGCGTTATTTTGTTG-3'
Sa1246RT	For 5'-ATGATAACACAGTGAGAGTTGAACC-3'
	Rev 5'-CTAACCCTTTGAAATCTTGCGTTG-3'
Sa1583RT	For 5'-TCAGCGAGATTGAAAGCGAATAC-3'
	Rev 5'-CTGTCCATTTCTTTAAGCGTCATAG-3'
Sa1844RT	For 5'-GTGAAATTCGTAAGCATGACCCAGTTG-3'
	Rev 5'-TGTAAGCGTGTATGTGCAGTTTCTAAAC-3'
Sa2174RT	For 5'- TGGAACACTGACACCATTAC
	Rev 5'- CTGATGCTTCTCGTTCTGAA
Sa1007RT	For 5'-CAACTGATAAAAAAGTAGGCTGGAAAGTGAT-3'
	Rev 5'-CTGGTGAAAACCCTGAAGATAATAGAG-3'
16S rRNART	For 5'-CTGTGCACATCTTGACGGTA-3'
	Rev 5'-TCAGCGTCAGTTACAGACCA-3'
PhlaT-Gfor	5'-GTTAATTTTTATTTAATAGTTAATTAATTGATTTA-3'
PhlaT-Grev	5'-TAAATCAATTAATTAACTATTAAATAAAAATTAAC-3'
PhlaTC-ATfor	5'-GATATTTCTATGTAATGGCAAAATTTATTCCCG-3'
PhlaTC-ATrev	5'-CGGGAATAAATTTTGCCATTACATAGAAATATC-3'
PhlaFor*EcoR*I	5'-ATGAATTCTTTAATCCCATATCACATTT-3'
PhlaRev*Xma*I	5'-TACCCGGGTTTCATCATCCTTCTATTTT-3'
Phla217for	5'-BiosgGCCTCTAACTAAAAACCTAC-3'
Phlarev482	5'-GTAATCGATTACAATATAAAAATAC-3'
Sa2174Ndelfor1	5'-TTCATATGATGTATGTAGAAAACAGCTATC-3'
Sa1274ZXholrev	5'-TTCTCGAGCTTTCTGTCGGAATAGTC-3'

2.4 Results

2.4.1 Hyperproduction of α-toxin in bovine mastitis *S. aureus* isolates, including RF122.

The comparative genomic analysis of human and bovine *S. aureus* isolates suggested that a unique mechanism may be involved in the pathogenicity of bovine *S. aureus* isolates (183). Numerous studies have demonstrated that staphylococcal exported proteins, especially toxins, are important virulence factors in bovine mastitis (178, 179). To examine whether human and bovine *S. aureus* isolates produce distinct levels of toxins, we conducted hemolytic assays using a sheep blood agar plate. The supernatants from the overnight cultures of human isolates, WCUH29 and COL strains, had dramatically less hemolytic activity compared to that of bovine isolate RF122 (Fig. 2-1A). To further determine whether there are different exported protein profiles between human and bovine *S. aureus* isolates, we chose human clinical isolates, including WCUH29, different strains of MRSA and twelve bovine *S. aureus* isolates, including the RF122 strain, to compare their exported proteins using SDS-PAGE. None of the human isolates produced dramatic amounts of α-toxin (Fig. 2-1B). In contrast, seven bovine strains, including RF122, exhibited extremely high levels of α-toxin production (Fig. 2-1C), although five bovine strains produced α-toxin at levels similar to the human isolates (Fig. 2-1D). To further confirm the identity of the over-expressed protein, we conducted a MALDI mass spectrometry assay and identified that the highly expressed protein is α-toxin (data not shown). Furthermore, qPCR analysis identified that RF122 has an 8-fold higher level of

hla transcript compared to WCUH29, suggesting the up-regulation of α-toxin is at the transcription level (Table 2-3).

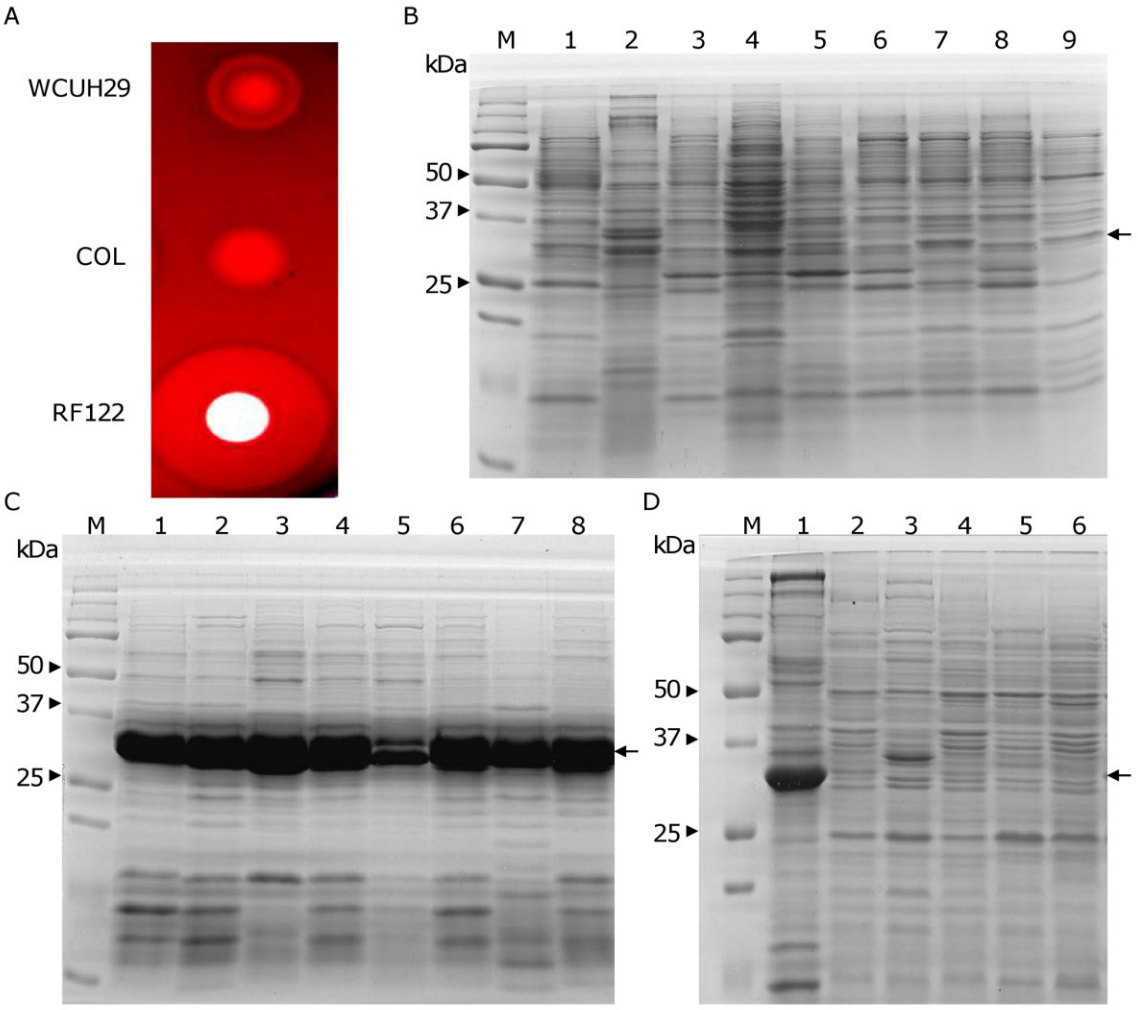

Figure 2-1 Hemolytic analysis and SDS-PAGE of the expression profiles of exported proteins. (A) Hemolytic analysis on Sheep Blood Agar. SDS-PAGE analysis of the expression profiles of the exported proteins. **(B)** Human *S. aureus* isolates, Lane 1, WCUH29; Lane 2, NRS105; Lane 3, NRS194; Lane 4, NRS 237; Lane 5, NRS243; Lane 6, NRS248; Lane 7, NRS384; Lane 8, MW2(956); Lane 9, USA300(1371); M, Precision Plus Protein Standard. **(C)** Alpha-toxin hyper-producing bovine mastitis *S. aureus* isolates. Lane 1, RF122; Lane 2, BSa39; Lane 3, BSa55; Lane 4, BSa60; Lane 5, BSa67; Lane 6, BSa68; Lane 7, BSa74; Lane 8, BSa97. M, Precision Plus Protein Standard. **(D)** Bovine mastitis *S. aureus* isolates. Lane 1, RF122; Lane 2, BSa12; Lane 3, BSa22; Lane 4, BSa28; Lane 5, BSa83; Lane 6, BSa110. M, Precision Plus Protein Standard. Arrow indicates α-toxin.

Table 2-3 Comparison of gene expression by qPCR analysis between the RF122 and WCUH29 strains

ORF (N315)	Gene	Fold change (RF122/WCUH29)
Sa1007	*hla*	8
Sa2174	*sarZ*	3
Sa1246	*arlS*	4
Sa0660	*saeS*	8
Sa1844	*agrA*	32
Sa1583	*rot*	-2
16SrRNA		0

2.4.2 Identification of single nucleotide polymorphisms (SNPs) in the *hla* promoter region.

To elucidate the potential mechanisms involved in up-regulating *hla* expression in the bovine *S. aureus* isolate RF122, we performed alignment analyses of *hla* promoter region based upon the published *S. aureus* genomes in the NCBI genome database. We found that the DNA sequences of *hla* promoter region are almost identical among the human *S. aureus* isolates (Fig. 2-2A). However, several nucleotides of the *hla* promoter region of bovine RF122 in the positions -484, -483 and -376 from the start codon are different from those of human isolates (Fig. 2-2A). To further investigate whether these differences exist in other human and bovine isolates, we isolated the chromosomal DNA from these isolates and the *hla* promoter region was amplified by PCR for sequencing. The DNA sequencing results showed that among seven α-toxin over-expressing *S. aureus* isolates, six isolates possess the same *hla* promoter DNA sequence as RF122 (Fig. 2-2B), indicating that these bovine isolates have predominant SNPs in the *hla* promoter region. In contrast, the *hla* promoter sequences of the five α-toxin hypoproduction strains were identical to the human *S. aureus* isolates (Fig. 2-2B). In addition, the DNA sequences of *hla* promoter region from eight human isolates were identical to the published human isolates (data not shown).

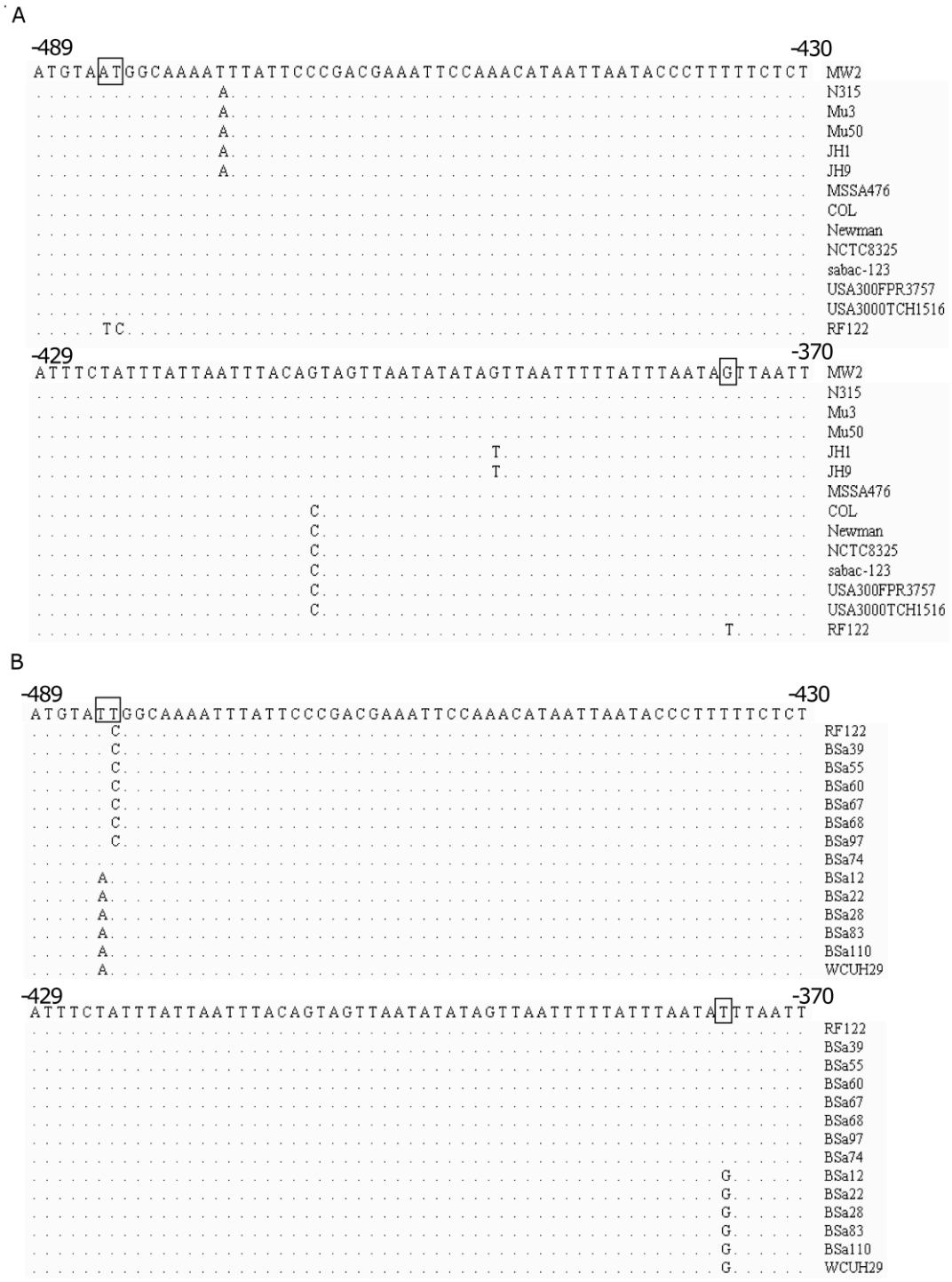

Figure 2-2 Alignments of bovine and human *S. aureus hla* promoters. Structural alignments with homologues of the *hla* promoter region from sequenced bovine and published human *S. aureus* genomes. The symbol "–" represents the upstream region from the start codon of *hla*. The boxed nucleotide represents the major nucleotides of difference between bovine *S. aureus* isolates and the human *S. aureus* isolate. **(A)** Human *S. aureus* isolates **(B)** Bovine mastitis *S. aureus* isolates.

2.4.3 The SNPs in the *hla* promoter region affects *hla* transcription.

The identification of predominant SNPs in the *hla* promoter region led us to hypothesize that the SNPs may be associated with the modulation of *hla* transcription. To test this possibility, we created both the human and bovine *S. aureus hla* promoter-*gfp-luxABCDE* dual reporter constructs in the human isolate WCUH29 and determined the *hla* expression levels by measuring bioluminescence intensity. The dual reporter construct carrying the RF122 *hla* promoter showed a significantly higher level of *hla* transcription than the dual reporter construct carrying the WCUH29 *hla* promoter (Fig. 2-3). The data suggests that the predominant SNPs in the RF122 *hla* promoter region are likely involved in the transcriptional modulation of *hla* expression.

To confirm this finding, we first performed site-directed mutations in the *hla* promoter region using the RF122 *hla* promoter-*gfp-luxABCDE* dual reporter construct. The site mutations were confirmed by DNA sequencing. Then, we determined the impact of the nucleotide mutations on growth and *hla* expression by monitoring *lux* expression levels in the WCUH29 strain. The mutations in the *hla* promoter region had no impact on growth (data not shown). However, the nucleotide mutations in either -376 (T→G) or -484 and -483 (TC→AT) in the RF122 *hla* promoter region significantly decreased the reporter gene expression level compared to that of the parental promoter (Fig. 2.3). Furthermore, the double mutations (T→G/TC→AT) in the RF122 *hla* promoter region dramatically diminished the reporter gene expression, which was comparable to the construct carrying the WCUH29 *hla* promoter (Fig. 2-3).

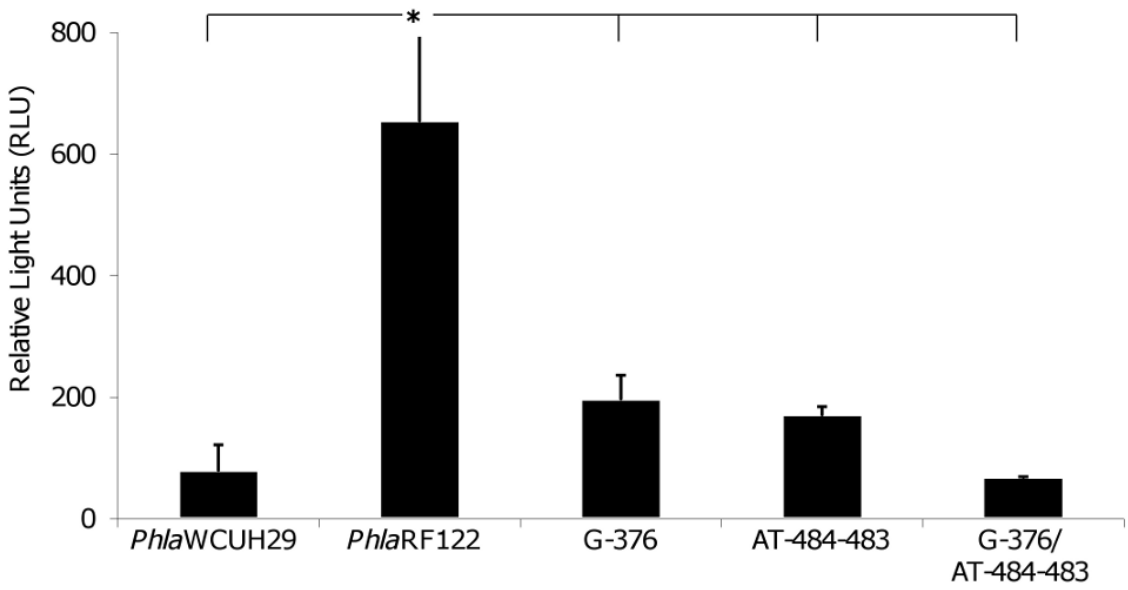

Figure 2-3 Influence of SNPs *hla* promoter-*luxABCDE* reporters on bioluminescence intensity of *S. aureus* WCUH29.
The maximal light intensity values are given as relative light units (RLU). The symbol "*" indicates a significant difference (P ≤ 0.05) between SaWH1207 and all other strains.

To further confirm the role of the SNPs of the *hla* promoter region in controlling the *hla* over-expression in RF122 strain, we electroporated the above *hla* promoter-*gfp*-*lux* dual reporter constructs into the RF122 strain and examined the impact of the site-directed mutations in the *hla* promoter region on reporter gene expression. Surprisingly, no bioluminescence signal was detectable in the cultures of these strains. However, we found that the introduction of either the RF122 *hla* promoter-*gfp*-*lux* dual reporter or either of the single (T→G, TC→AT) mutated *hla* promoter-*gfp*-*lux* dual reporters into the RF122 strain either eliminated or severely reduced endogenous *hla* expression, respectively, indicating that dominant-negative effects occurred in the strains (Fig. 2-4A, lane 2, 3 and 4). In contrast, the introduction of the WCUH29 *hla* promoter-*gfp*-*lux* reporter and the double mutated (T→G/TC→AT) RF122 *hla* promoter-*gfp*-*lux*

reporter did not have an appreciable effect on endogenous *hla* expression (Fig. 2-4A, lane 5 and 6). In addition, we determined the impact of the SNPs of *hla* promoter region on reporter *gfp* expression using a western blotting assay. An intense band of Gfp was exhibited in the whole cell lysates of the reporter construct carrying the wild-type RF122 *hla* promoter; contrastingly, either no band or a weak band of Gfp was detected in the whole cell lysates of constructs carrying either the WCUH29 *hla* promoter or the double (T→G/TC→AT) mutated RF122 *hla* promoter (Fig. 2-4B). Both the T→G and the TC→AT mutations in the RF122 *hla* promoter led to a 2- to 3-fold decrease of *gfp* expression (Fig. 2-4B). Taken together, the above data demonstrate that the SNPs in the *hla* promoter region participate in the modulation of *hla* expression in the bovine *S. aureus* RF122 isolate.

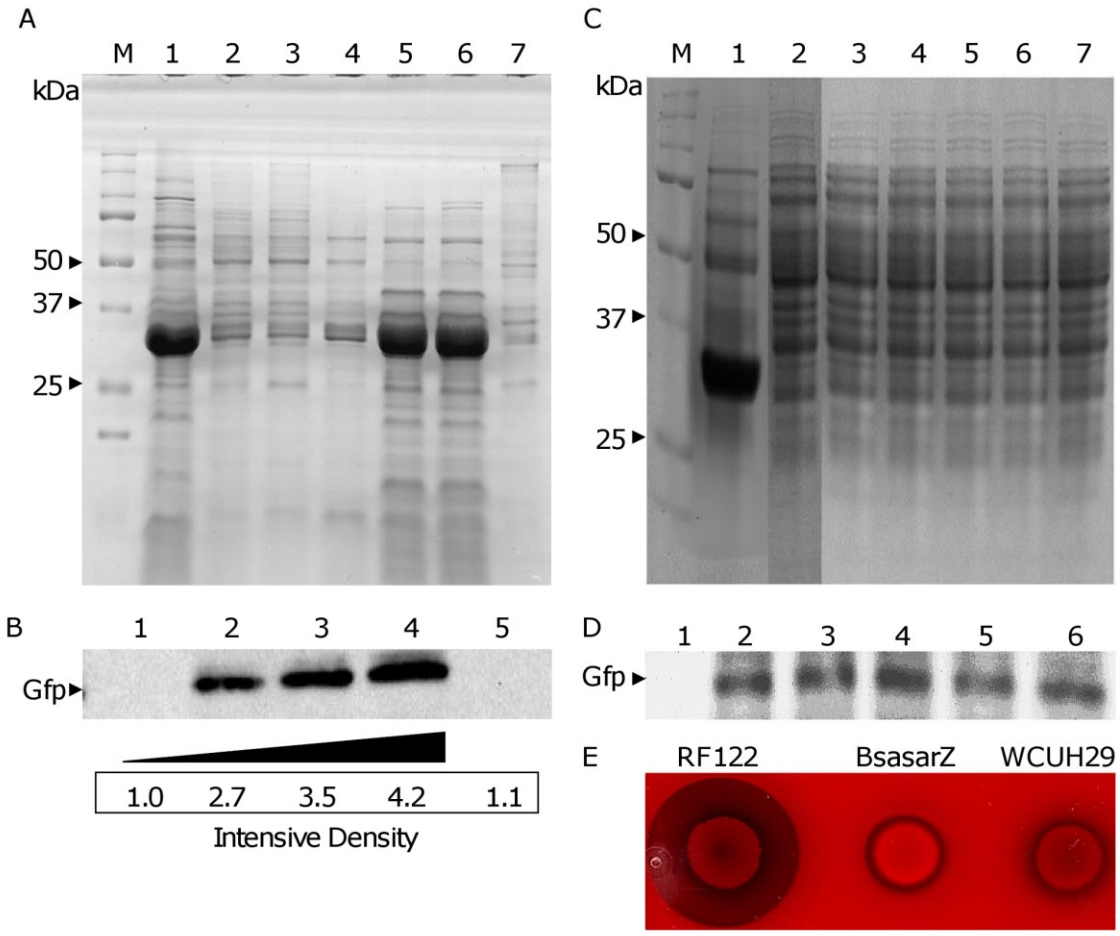

Figure 2-4 Impact of *hla* promoter reporter and nucleotide mutations on alpha-toxin and GFP expression in RF122 strain.
(A) SDS-PAGE analysis of *hla* expression of the *S. aureus* RF122 strain carrying different *hla* promoter-*gfp* fusions (P*hla*-fusions). Lane 1, RF122 control; Lane 2, SaRF1207 (RF122 P*hla*-fusion); Lane 3, SaRF1307 (G-376 mutated RF122 P*hla*-fusion); Lane 4, SaRF1407 (AT-484-483 mutated RF122 P*hla*-fusion); Lane 5, SaRF1507 (G-376 AT-484-483 mutated RF122 P*hla*-fusion); Lane 6, SaRF1107 (WCUH29 P*hla*-fusion); Lane 7, WCUH29 control. M, Precision Plus Protein Standard. **(B)** Western blot analysis of GFP expression from different P*hla*-fusions in RF122. Lane 1, SaRF1507; Lane 2, SaRF1307; Lane 3, SaRF1407; Lane 4, SaRF1207; Lane 5, SaRF1107. Black triangle indicates the increase of intensity of reaction band. **(C)** SDS-PAGE analysis of alpha-toxin expression of the *S. aureus* RF122 *sarZ* null mutant (BsasarZ). Lane 1, RF122 control, Lane 2, BsasarZ control, Lane3, BSasarZ1107; Lane 4, BSasarZ1207; Lane 5, BSasarZ1307; Lane 5, BSasarZ1407; Lane 7, BSasarZ1507; M, Precision Plus Protein Standard. **(D)** Western blot analysis of Gfp expression from different P*hla*-fusions in BSasarZ. Lane 1, RF122 wild-type control; Lane 2, BSasarZ1107; Lane 3, BSasarZ1207; Lane 4, BSasarZ1307; Lane 5, BSasarZ1407; Lane 6, BSasarZ1507. **(E)** Hemolytic assay on Sheep Blood Agar.

2.4.4 SarZ is associated with the SNPs in the regulation of hyperproduction of α-toxin.

In order to identify regulators that are involved in the SNPs' regulation, we employed DNA affinity purification using a biotinylated RF122 *hla* promoter region oligonucleotide bound to Dynabeads M-280 Strepavidin coated paramagnetic beads. The cytoplasmic proteins of RF122 specifically binding to the *hla* promoter region oligonucleotide were eluted from the beads and separated by SDS-PAGE (data not shown). To determine the identity of *hla* promoter region binding proteins, we employed MALDI mass spectrometry assays and revealed SarZ and Mgr bound to the *hla* promoter region of RF122 (data not shown).

To confirm whether SarZ protein is associated with the SNPs in the modulation of *hla* expression genetically, we constructed a *sarZ* null mutant in the RF122 strain by phage transduction. The *sarZ* null mutant, BSasarZ, was confirmed by diagnostic PCR and DNA sequencing. The five individual *hla* promoter-*gfp*-*lux* reporter vectors were introduced into BSasarZ by electroporation. First, we examined how the loss of SarZ affected the endogenous *hla* expression by using a Sheep Blood Agar hemolysis assay and SDS-PAGE and found that the *sarZ* null mutation abrogates the α-toxin hyper-production phenotype of RF122 (Fig. 2-4C and E). Furthermore, the *sarZ* mutation eliminated the dominant-negative effects of the RF122 *hla* promoter-*gfp*-*lux* dual reporter (Fig. 2-4C) compared to the wild-type strains (Fig. 2-4A). Next, we determined the impact of SarZ on reporter *gfp* expression using a

western blotting assay. In contrast to the RF122 wild-type strain (Fig. 2-4B), no obvious difference of *gfp* expression was revealed in the whole cell lysates of *sarZ* mutants carrying the WCUH29 *hla* promoter, the single (T→G or TC→AT) mutated RF122 *hla* promoter, the double (T→G/TC→AT) mutated RF122 *hla* promoter or the wild-type RF122 *hla* promoter (Fig. 2-4D).

To further confirm the specific effect of SarZ and identify binding affinity differences, we performed gel-shift assays. The PCR products were purified and labeled using DIG. The recombinant His-tagged SarZ protein was purified from *E. coli*, and was shown to be more than 95% pure as detected by SDS-PAGE (Fig. 2-5A). The gel-shift assays showed that the addition of as little as 100 ng of SarZ protein could retard the electrophoretic mobility of RF122 *hla* promoter region DNA (Fig. 2-5B), whereas approximately 500 ng of SarZ was required to retard the electrophoretic mobility of the WCUH29 *hla* promoter region DNA (Fig. 2-5C). The shifted complexes of DNA-SarZ protein exhibited predominant bands when the amounts of SarZ for both *hla* probes was increased, whereas addition of excess amounts of the same, but unlabeled, *hla* promoter region (specific competitor) eliminated the electrophoretic mobility shift (Fig. 2-5B and C). These results indicate that the RF122 *hla* promoter binding affinity of SarZ is higher than for the WCUH29 *hla* promoter region.

To examine whether the overproduction of α-toxin is also attributable to different levels of SarZ between human and bovine isolates, we performed real-time RT-PCR. We found that in the RF122 strain the transcriptional level of *sarZ* was 3-fold higher than that of the WCUH29 strain (Table 2-3). Taken together,

the above results suggest that the SNPs' regulation of *hla* expression may function through SarZ directly. Furthermore, it suggests the high level of α-toxin in the RF122 strain is a result of an increased transcription level of *sarZ* and increased binding affinity for the RF122 *hla* promoter by SarZ, leading to an α-toxin hyper-producing phenotype. We did not investigate the impact of Mgr, because the DNA-binding motif (GTTG, (168)) of Mgr is outside the SNPs region in the *hla* promoter region.

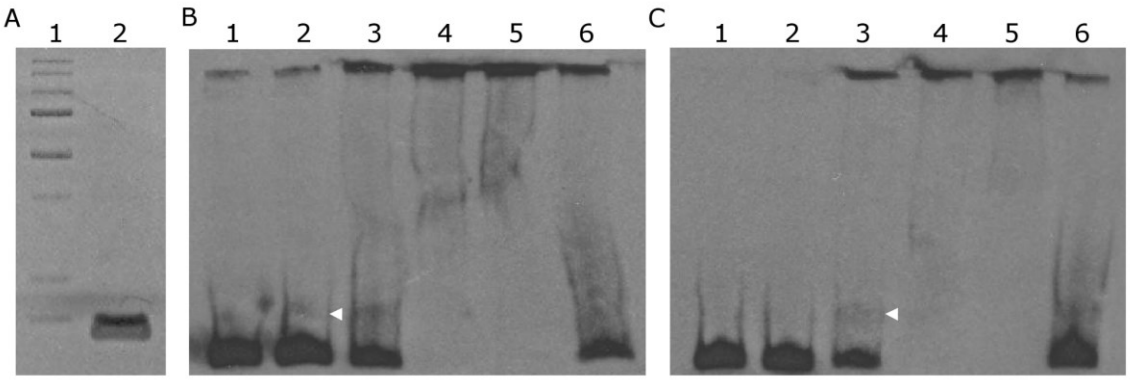

Figure 2-5 Gel-shift analysis of His-SarZ protein binding to the SNP containing region of the *hla* promoter. Lanes 1 to 5 correspond to 0, 0.1, 0.5, 1.25 and 1.75μg, respectively, of purified His-SarZ **(A)** mixed with DIG-labeled *hla* promoter region of RF122 **(B)** and WCUH29 **(C)**. Lane 6, the mixture of 1.75μg of purified His-SarZ protein with DIG-labeled *hla* probe and 100-fold excess of unlabeled specific competitor DNA. Head arrow represents the first detected mobility shift of each *hla* promoter region.

2.4.5 Involvement of *agr*, *arlRS*, *saeRS* and *rot* in the modulation of hyperproduction of α-toxin in the bovine *S. aureus* isolate RF122.

It is well known that different regulators, including two-component signal regulators, such as *agr, saeRS,* and *arlRS* systems, and transcriptional regulators such as *sarA* family proteins, such as *rot*, coordinately regulate *hla* expression (116, 162). Building off of our previous finding that RF122 has a higher *sarZ* transcript level, we hypothesized the hyper-production of α-toxin may also be attributable to differential expression of these regulators between the bovine *S. aureus* RF122 and the human *S. aureus* WCUH29. To address this question, we examined the transcriptional levels of selected *hla* regulators using qPCR. The real-time RT-PCR results are shown in Table 2-3. In the bovine *S. aureus* RF122 strain, the transcriptional levels of *hla* positive regulator genes *agr*, *saeRS* and *arlRS* were higher than those in the human WCUH29 strain, whereas the transcriptional level of *hla* repressor gene *rot* was lower than that in the human WCUH29 strain (Table 2-3). The data suggest that the over-transcription of the *hla* positive regulators *agr*, *arlRS,* and *saeRS* and decreased transcription of the *hla* negative regulator *rot* also, at least in part, contributes to the α-toxin hyper-production phenotype of RF122.

2.5 Discussion

Our studies revealed that the *hla* promoter region possesses predominant SNPs that contribute to the modulation of *hla* over-expression in some bovine *S. aureus* isolates. Although no specific SNP is identified in the *hla* promoter region of the human *S. aureus* isolates tested, we cannot exclude the possibility that SNPs may exist in the *hla* promoter region in other human *S. aureus* isolates. Notably, we identified that a known *hla* transcriptional regulator, SarZ, has a higher binding affinity for the SNPs region and contributes to the involvement of SNPs in regulating *hla* expression. Moreover, we revealed that the transcription level of *sarZ* is higher in the bovine *S. aureus* RF122 strain than in the human WCUH29 strain. This suggests that the higher transcription level of *sarZ* is, at least in part, involved in the α-toxin hyper-production phenotype of the RF122 strain. Importantly, we also demonstrated that the SNPs contribute to the regulation of *hla* regardless of the genetic background, WCUH29 or RF122, in that the RF122 *hla* promoter-*gfp*-*lux* dual reporter construct had higher levels of expression in both backgrounds compared to the WCUH29 *hla* promoter-*gfp*-*lux* dual reporter construct. Additionally, the site-directed mutation of the RF122 promoter's SNPs progressively decreased reporter expression in both backgrounds (Fig. 2-3 and 2-4B). Our findings are partially supported by a previous report that ST151 strains, including the RF122 strain, have elevated levels of *hla* and RNAIII expression (135). Taken together, these findings indicate that unique mechanisms may be involved in the up-regulation of *hla* expression

in bovine *S. aureus* isolates, which in turn may partially contribute to the pathogenicity in bovine mastitis.

DNA sequencing analysis indicates that there are predominant SNPs in the *hla* promoter region of bovine *S. aureus* isolates, which occur at positions -376, -483 and -484 upstream, of the *hla* start codon. In the α-toxin hyperproduction isolates, there are apparent nucleotide shifts from G to T and T to C at positions -376 and -483, respectively, compared with the α-toxin hypoproduction isolates. These nucleotide shifts lead to a decreased binding affinity of SarZ (Fig. 2-5), which is consistent with a decreased *hla* expression level of the WCUH29 *hla* promoter-*gfp* fusion reporter system (Fig. 2-4B). It has been reported that the DNA-binding protein, SarZ, regulates *hla* expression by binding to the *hla* promoter region (169, 170), and is associated with the pathogenicity of *S. aureus* (169). Furthermore, the introduction of RF122 *hla* promoter-reporter fusion into the RF122 strain led to a dominant-negative effect on endogenous *hla* expression; this effect was eliminated in a *sarZ* null background. Taken together, the above data indicate that the SarZ protein has a higher binding affinity for the RF122 *hla* promoter and is directly involved in differential regulation of *hla* expression through SNPs, which in turn may contribute to virulence of some genetically similar bovine *S. aureus*. Our results are similar to previous findings that a single nucleotide (T→C) mutation at position -215 bp in the promoter region of the nitrate reductase operon *narGHJI* in *Mycobacterium tuberculosis* and *Mycobacterium bovis* leads to differential activity of reductase and altered virulence capacity (83); and that in *Vibrio*

cholerae, nucleotide differences at positions -65 and -66 bp in the *tcpPH* (encoding a toxin-coregulated pilus transcriptional activator pair, TcpPH) promoter region is not only responsible for determining the classical and EL Tor biotypes, but also contributes to differential regulation of virulence gene expression through a *tcpPH* regulator, AphB (82). Although we found that the DNA binding motif of Mgr is not located in the SNPs, we cannot exclude the possibility that Mgr may contribute to the hyperproduction of α-toxin, because *sarZ* is transcriptionally regulated by Mgr (170).

It was reported that the ET3 clone is predominant in bovine mastitis *S. aureus* isolates (184) and that different subtypes exhibit different expression levels of α-toxin in the ET3 clone (135). The RF122 strain belongs to the ET3 clone ST151 subtype (135); therefore, it is necessary to determine whether bovine *S. aureus* isolates that possess the predominant SNPs in the *hla* promoter region belong to the common bovine *S. aureus* clone (ET3) and/or the same subtype within the ET3 clone. The results would allow us to evaluate whether the identified predominant SNPs of the *hla* promoter region would be useful as a target for molecular diagnosis of *S. aureus* isolates that posses an α-toxin hyper-productive phenotype and may cause severe bovine mastitis.

The *agr* locus in *S. aureus* has been examined for its role in the expression of exotoxins and cell surface proteins (185). It has been reported that variations in α-toxin production of *S. aureus* isolates from humans and bovines were due to variations of the RNA III transcript in *agr* locus (148, 186). Our data also revealed that in the RF122 strain the transcriptional levels of *hla* positive

regulators genes *agr, saeR* and *arlR* are higher in the bovine *S. aureus* RF122 strain than in the human WCUH29 isolate in the same culture medium; however, the transcription level of the *hla* repressor gene *rot* is lower in the RF122 strain. Although the SNPs are located outside of the *hla* promoter DNA-binding regions (GEEAAN$_6$GTTAA from -405 to -390 or TTTAAN$_6$GTTA from -190 to -175) of phosphorylated SaeR (94) and the DNA-binding motif of Mgr (170), to define the mechanisms of their regulation is beyond the scope of this study. In addition, we found that the impact of SNPs on *hla* expression in the sigma B deficient strain RN4220 (Figure 2-6) is the same as in the sigma B positive strains WCUH29 and RF122. Thus, we can exclude the potential effect of sigma B factor on the SNPs' involvement in regulating *hla* expression, although sigma B factor is associated with mediating *hla* expression (174, 175).

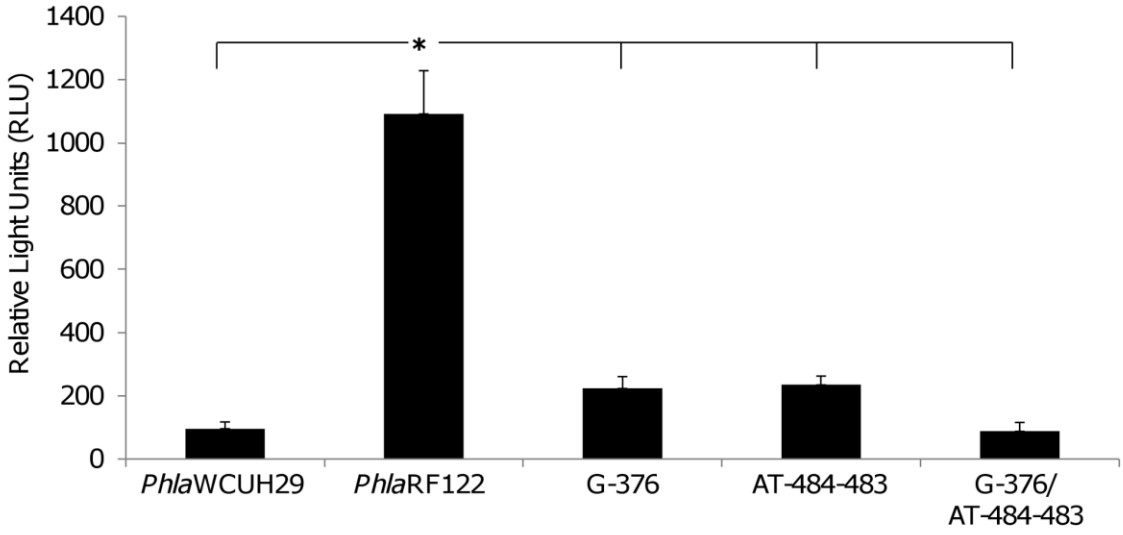

Figure 2-6 Influence of SNPs hla promoter-*luxABCDE* reporters on bioluminescence intensity of *S. aureus* RN4220.
The maximal light intensity values are given as relative light units (RLU). The symbol "*" indicates a significant difference (P ≤ 0.05) between SaRN1207 and all other strains.

It was previously reported that the RF122 strain belongs to ST151 (a subtype of ET3 clone) (183). Our unpublished data showed that the supernatant of RF122 culture is more toxic than the supernatant of WCUH29 using MAC-T cells (187). This is consistent with recent findings that among ET3 subtypes, the ST151 strain is more virulent in an intramammary gland infection (135). Previous studies have also demonstrated that most *S. aureus* isolates from the mammary gland of dairy cows produce α-toxin (179). Significantly higher amounts of antibodies against both α- and β-toxins are exhibited in milk isolated from cows with chronic staphylococcal mastitis (178), and immunization of animals with attenuated α-toxin protects from *S. aureus*-induced mastitis (188). Taken together, the above data suggest that the hyperproduction of α-toxin is a probable key factor for *S. aureus* to cause severe cow mastitis, although we cannot rule out the importance of other virulence factors in the pathogenesis of bovine mastitis (189).

In conclusion, we are the first to identify SNPs in the *hla* promoter region that results in the hyperproduction of α-toxin in many bovine *S. aureus* isolates. Importantly, we have identified and demonstrated that the DNA-binding protein SarZ has a higher binding affinity for the RF122 *hla* promoter region containing the SNPs and is transcribed at higher levels in the RF122 strain, thus contributing to the involvement of SNPs in differential regulation of *hla* expression. In addition, we found that the over-expression of *agrA*, *arlR* and *saeR*, and the down-regulation of *rot*, may partially contribute to the hyperproduction of α-toxin in the RF122 strain.

Chapter 3 : Characterization of the *Staphylococcus aureus yhcSR* two-component signal transduction system

3.1 Overview

Two-component signal transduction systems (TCSs) are important for adapting gene expression in response to extrinsic stimuli. Staphylococcal TCSs have a role in regulating a variety of pathways including cell-wall biosynthesis, biofilm development and virulence factor expression. The *Staphylococcus aureus yhcSR* two-component signal transduction system was previously shown to be essential in the *S. aureus* RN4220 laboratory strain and expression of *yhcS* antisense RNA inhibited growth of the HA-MRSA WCUH29 strain. As an extension of this previously published work, the *yhcSR* TCS was found to be absolutely essential for aerobic and anaerobic viability of *S. aureus* WCUH29. Furthermore, expression of *yhcS* antisense RNA in the CA-MRSA 923 and MSSA Newman strains inhibited bacterial growth. Additionally, the expression of *yhcSR* was dramatically up-regulated in the absence of oxygen and the importance for production of YhcSR in the absence of oxygen was highlighted by the inability of *S. aureus* to perform dissimilatory nitrate reduction during *yhcS* antisense RNA expression. Lastly, the YhcSR TCS may be involved in regulation of the cellular division or cell-wall biosynthesis. Depletion of YhcSR resulted in slower growth and abnormally large cells. Overproduction of the response regulator, YhcR, produced uniformly enlarged cells with possible cell-wall thickening as visualized by staining. The YhcSR TCS is essential for *S. aureus* growth and the essentiality is likely related to regulation of cellular metabolic pathways.

3.2 Introduction

Staphylococcus aureus possesses many genes that allow the organism to sense and adapt to ever-changing environmental conditions. At least 16 operons encode two-component signal transduction systems (TCSs) in *S. aureus* (17), with 14 conserved in all sequenced genomes (91). The TCSs sense a variety environmental conditions and manipulate cellular responses accordingly. A prototypical TCS consists of a dimerized sensor histidine kinase (HK) that responds to a specific environmental cue(s) by transautophosphorylating and subsequently transferring the phosphate to a cognate response regulator. Phosphorylation of the response regulator activates the effector function of the protein. The effector function ultimately leads to modulation of gene expression within the TCS regulon, mediating the cellular response to the environmental cue (102–105).

The novel *yhcSR* TCS of *S. aureus* was found to be essential in a laboratory strain of *S. aureus* and depletion of YhcSR by *yhcS* antisense RNA inhibited growth of the clinically relevant hospital-acquired methicillin-resistant *S. aureus* WCUH29 strain (HA-MRSA) (128). This information was the foundation for the continued characterization of this TCS as well as the fact the *yhcSR* TCS appears to be conserved with at least 40% conserved identity in the GAF sensory domain of YhcS throughout the Bacilli Class. Definitively, using BLASTP analysis, the cysteine residues that are essential for oxygen sensing by YhcS (129) are conserved in all identified homologues in the Bacilli Class. This conservation suggests the YhcSR TCS is important for oxygen sensing within

Bacilli, but more importantly, the YhcSR TCS may be essential in other pathogens such as *Bacillus anthracis* and constitutes a novel target for the development of new antibiotics.

Little was known about the functional role of YhcSR when this work began. The only confirmed genes to be regulated by YhcSR were involved in dissimilatory nitrate reduction, *narGHIJ* and *nreABC* (100) and it provided the background for confirming the essentiality and determining the temporal expression pattern of *yhcSR* in *S. aureus* WCUH29 aerobically and anaerobically. As a way to gain insight into the function of YhcSR the cellular phenotype of *S. aureus* YhcR producing and YhcSR deplete cells were studied which revealed changes in cellular morphology.

3.3 Materials and methods

3.3.1 Bacterial strains, plasmids and growth media.

The bacterial strains and plasmids used in this study are listed in Table 3-1. The *S. aureus* strains were cultured in trypticase soy broth (TSB) at 37˚C with shaking. *E. coli* strains were cultured in Luria-Bertani (LB) broth. Transformants containing recombinant plasmids were selected on LB agar containing spectinomycin (100 μg/ml) for *E. coli*, and trypticase soy agar (TSA) containing chloramphenicol (10 μg/ml), tetracycline (5 μg/ml), or erythromycin (5 μg/ml) for *S. aureus*. All plasmids were constructed in *E. coli* then purified and electroporated into *S. aureus* RN4220 and subsequently electroporated into the relevant *S. aureus* strain.

3.3.2 Construction of the *S. aureus* Newman and 923 *yhcS* antisense RNA strain.

S. aureus Newman and *S. aureus* 923 were electroporated with the control plasmid, pCY406, or the *yhcS* antisense RNA plasmid, pCY606. Overnight cultures of *S. aureus* strains were diluted to ~10^4 CFU/ml with TSB containing 5 μg/ml of erythromycin and different concentrations of an inducer anhydrotetracycline, (ATc), at concentrations of 0, 100, 250 ng/ml. Cell growth was monitored at 37ºC by measuring the optical density at 600 nm ($OD600_{nm}$) every 15 min, with 1 min of mixing before each reading in a BioTek Synergy II microplate reader.

3.3.3 Construction of *S. aureus* WCUH29::P*spac-yhcSR* (JH312).

Using the primers YhcS For pLH1-NotI RBS/YhcR Rev pLH1-BamHI the *yhcSR* operon was obtained by high fidelity PCR, purified, and digested with *Not*I and *BamH*I (NEB). The plasmid pLH1 was digested with the same enzymes and the PCR product was ligated into pLH1 with T4 DNA ligase (Promega). Correct insertions were confirmed by diagnostic PCR.

To create strain JH312, the plasmid p312 was electroporated into *S. aureus* strain CYL557 (190) and transductants were plated on TSA containing tetracycline, chloramphenicol, and 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). Insertion of plasmid p312 into the chromosomal φ11 integration site to create strain RN312 was confirmed by diagnostic PCR using the primer sets scv4 paired with scv10 and primer scv8 paired with scv9 listed in Table 3-2. After confirmation of insertion, one of the RN312 transductants was used to make a phage lysate by using *S. aureus* phage φ11. The phage lysate was then used to infect WCUH29 as described (159) and tetracycline resistance was used to select for potential transductants. Diagnostic PCR using the primers sets scv4/scv10 and scv8/scv9 were used to confirm the transduction event.

3.3.4 Construction of an in-frame *yhcSR* deletion plasmid.

Using the *yhcS/yhcR* pKOR1 primers listed in Table 3-2 approximate 1 kB DNA regions upstream and downstream of *yhcSR* was amplified by PCR. Each PCR product was gel purified. A 5' phosphate was attached to the downstream segment's forward primer during oligonucleotide synthesis. Equal molar concentrations of upstream and downstream PCR segments were ligated

together overnight utilizing the 5' phosphate on the downstream PCR product. Five µl of the ligation reaction was mixed with BP Clonase II, TE buffer, and plasmid pKOR1 and incubated overnight at 25°C. The following day the pKOR1 reaction was transformed using DH10B *E. coli* with selection on LB agar with ampicillin for selection of recombinants (191). DH10B *E. coli* was grown at 30°C. Recombinant plasmids were confirmed by PCR and electroporated into *S. aureus* RN4220 and incubated at the replication-permissive 30°C temperature. A RN4220 transformant was cultured at 30°C overnight and the pKOR1-Δ*yhcSR* plasmid was purified from RN4220.

3.3.5 Deletion of endogenous *yhcSR* locus.

After the *Pspac-yhcSR* segment was transduced into the WCUH29 chromosome, an attempt was made to delete the endogenous *yhcSR* operon by homologous recombination using the temperature-sensitive plasmid pKOR1 as described (191). The pKOR1-Δ*yhcSR* plasmid was electroporated into wild-type *S. aureus* WCUH29 or JH312 using chloramphenicol (and tetracycline for JH312) for selection and incubated at 30°C overnight. A single colony from each of the resulting plates was cultured in TSB with appropriate antibiotics at 30°C overnight. The following day 5 µl of each overnight culture was used to inoculate separate, pre-warmed, TSB with appropriate antibiotics for each strain and incubated overnight at 42°C; the plasmid's non-replication permissive temperature. 50 µl of each culture was streaked onto TSA containing the appropriate antibiotics and incubated overnight at 42°C. The following day, a single large colony from each plate was cultured overnight in TSB at 30°C and

subsequently sub-cultured two more times. To induce the second homologous recombination event and loss of the pKOR1 plasmid backbone, the third day sub-culture of each strain was serially diluted to ~10^{-4} CFU/ml and 50 µl of each culture was plated on TSA and TSA containing 200 ng/ml of anhydrotetracycline (tetracycline was included for selection of JH312). One set of plates, consisting of WCUH29 WT and JH213, was incubated aerobically overnight at 37°C. A second set of plates was incubated in an anaerobic COY chamber (O_2-free, N_2-He mix) between 25-30°C for 4 days. Randomly chosen large colonies from the TSA/ATc plates were screened by colony PCR using the primer set attB1-yhcS-L-F-pKOR1/attB2-yhcR-R-R-pKOR1 for aerobic plates and the primer set Yhc KO For/Yhc KO Rev for the anaerobically grown plates. See Table 3-2 for primer sequences. GoTaq Green PCR mix (Promega) was used for colony PCR screening. Colonies were suspended in 10 mM Tris-Cl, pH 7.5 containing 50 µg/ml lysostaphin and incubated at 37°C for at least 30 minutes to weaken the cell wall. One microliter of the suspension was used as the PCR template.

3.3.6 Construction and monitoring of P*yhc-luxABCDE* reporter plasmid.

The promoter region of *yhcSR* was obtained and cloned upstream of the a promoterless *luxABCDE* cassette creating plasmid pCY106 (100). The plasmid was electroporated into RN4220 and subsequently into WCUH29. The overnight culture of *S. aureus* strain YJ106 was diluted to ~10^4 CFU/ml with TSB containing appropriate antibiotics and in the absence or presence of 5.7 mM cysteine as a free oxygen scavenger. The bioluminescence signal and cell growth were monitored at 37ºC by measuring the light intensity and optical

density at 600 nm ($OD600_{nm}$) with a BioTek Synergy II spectrophotometer every 30 min. To eliminate the effect of bacterial growth on light intensity, the relative light units (RLU) were calculated (light intensity/$OD600_{nm}$) from duplicate readings at different times during growth. The data represent the mean RLU and $OD600_{nm}$ of three independent colonies.

3.3.7 RNA isolation and purification.

An overnight culture of *S. aureus* WCUH29 was inoculated at 1% in TSB medium with appropriate antibiotics. Aerobic cultures were grown in an Erlenmeyer flask with the medium making up no more than 5% of the total flask volume and with vigorous shaking at 37°C. Anaerobic cultures were grown in sealed TSB filled Wheaton Serum bottles bubbled with oxygen-free nitrogen, autoclaved, and 5.7 mM fresh cysteine was added to remove traces of free oxygen. Each culture was grown to an $OD600_{nm}$ of 0.4, collected, and RNA extracted. Total RNA was purified from the above culture as described (192). Briefly, bacterial cells were harvested by centrifugation, and the RNA was isolated by the RNAPrep kit (Promega). Contaminating DNA was removed with a DNA-*free* kit (Ambion), and the RNA yield was determined spectrophotometrically at 260nm. The integrity of the RNA was checked by formaldehyde agarose electrophoresis and no degradation of the major 23S and 16S ribosomal RNA was detected.

3.3.8 Quantitative real-time RT-PCR (qPCR) analysis.

In order to determine if *yhcSR* is up-regulated during anaerobic growth quantitative real-time reverse transcription (RT) PCR was employed to compare

the RNA levels, as described (166, 192). The first strand cDNA was synthesized using the SensiFAST™ cDNA Synthesis Kit (Bioline) according to the manufacturer's protocol. 110 ng of total RNA was used for cDNA synthesis. Total cDNA was diluted 1:40 for *yhcS* qPCR and 1:1000 for 16S qPCR. For each RNA sample, duplicate reactions of reverse transcription were performed, as well as a control without reverse transcriptase, in order to determine the levels of DNA contamination. PCR reactions were set up in duplicate by using the PerfeCTa® SYBR© Green 2X Fastmix kit (Quanta Biosciences). Real-time sequence-specific detection and relative quantitation were performed with the Stratagene Mx3000P Real Time PCR System. Gene-specific primers were designed to yield ~100 bp of specific products (Table 3-2). Relative quantification of the product was calculated using the Comparative C_T method, as described for the Stratagene Mx3000P system. The housekeeping gene 16S rRNA was used as an endogenous control (166). All samples were analyzed in duplicate and normalized against 16S rRNA gene expression.

3.3.9 Analysis of conversation of nitrate to nitrite in YhcSR-depleted *S. aureus* WCUH29.

To measure nitrate conversion during *yhcS* antisense induction, overnight cultures of YJ406 and YJ606 were cultured overnight with appropriate antibiotics and each strain was washed with PBS and diluted 1:100 in CDM with 20 mM sodium nitrate, 5.7 mM cysteine as an oxygen scavenger, and 500 ng/ml of inducer, ATc. The conversion of nitrate (NO_3) to nitrite (NO_2) by *S. aureus* was measured using the Sigma Aldrich Griess Reagent Solution according to the

manufacturer's instructions and compared to empirical determined standard curve. The relative nitrite concentration is calculated by dividing the nitrite concentration by the $OD600_{nm}$ of the culture at each time point. The data represent the mean and standard deviation of 3 independent experiments.

To create the pKOR1-Δ*nreABC* plasmid, approximately 1 kb upstream and downstream of the *nreABC* locus was amplified by PCR using primers in Table 3-2, ligated together and cloned into the *S. aureus* allelic replacement plasmid, pKOR1 (191) and deletion of *nreABC* was carried out aerobically and confirmed by diagnostic PCR as described previously. To create the complementation plasmids, the *yhcSR* and *nreABC* operons were PCR amplified using primers YhcSOE-for/YhcROE-rev and NreABC-orf-PmeI-F/ NreABC-orf-AscI-R listed in Table 3-2, digested with *Asc*I and cloned downstream of the TetR regulated promoter in the pYH4 shuttle vector digested with *Pme*I and *Asc*I. Each plasmid was subsequently electroporated into *S. aureus* WCUH29.

3.3.10 Gram-stain, imaging and cell diameter measurements

Overnight cultures of each strain were cultured with inducer ATc, 500 ng/ml for antisense RNA control and JSAS909, and 250 ng/ml of ATc for overproduction control and WYhcR. 5 µl of each control and inducible mutant strain were Gram-stained using the Fisher Scientific Gram-stain kit according to the manufacturer's protocol. Images were taken at 100X magnification under oil immersion using an Olympus BX41 Microscope with an attached Olympus XC30 top-mounted camera and cellSens Software. The cell diameter of at least 200

individual cells from each image were measured using cellSens. Statistical and graphing analysis were conducted in Microsoft Excel.

Table 3-1 Bacterial strains and plasmids

Strain	Description	Source
DH10B	*E. coli* used for cloning and plasmid construction	Invitrogen
DB3.1	*E. coli* used for propagation of plasmid pKOR1	Invitrogen
RN4220	*S. aureus* Laboratory strain: *rsbU*$^-$	(181)
WCUH29	Clinical human *S. aureus* isolate: *rsbU*$^+$	(109)
Newman	Methicillin sensitive *S. aureus* strain with plasmid pCY406; Erm^r	This study
Newman-606	Newman with plasmid pCY606; Erm^C	This Study
MRSA923	Community-acquired methicillin resistant *S. aureus* stain with plasmid pCY406; Erm^C	This Study
MRSA923-606	MRSA923 with plasmid pCY606; Erm^r	This study
JSAS909	WCUH29 containing plasmid pSAS909 carrying *yhcS* antisense; Erm^r	(128)
CYL557	RN4220 that carries pLL2787 which encodes the φ11 *int* gene under the control of the IPTG inducible P*spac* promoter; Cm^r	(190)
YJ106	WCUH29 containing plasmid pCY106, Cmr	(100)
RN312	RN4220::P*spac-yhcSR*; Tet^r	This Study
JH312	WCUH29::P*spac-yhcSR*; Tet^r	This Study
YJ406	WCUH29 with pCY406; Erm^r	(100)
YJ606	WCUH29 with pCY606; Erm^r	(100)
WYhcSR	WCUH29 with pYhcSR; Erm^r	This Study
WNreABC	WCUH29 with pNreABC; Erm^r	This Study
WCUH29Δ*nreABC*	WCUH29 with in-frame chromosomal deletion of *nreABC*	This Study
JHYhcSR	WCUH29Δ*nreABC* with pYhcSR; Erm^r	This Study
JHNreABC	WCUH29Δ*nreABC* with pNreABC; Erm^r	This Study

Table 3-2 Plasmids

Plasmids	Description	Source
pCY406	Shuttle vector, derived from pYH4, containing *luxABCDE*; Erm^r	(193)
pCY606	Shuttle vector, derived from pSAS909, containing *luxABCDE* and *yhcS* antisense; Erm^r	(193)
pCY106	*yhcSR* promoter-*luxABCDE* reporter system, Cm^r	(100)
pYH3	Shuttle vector with a TetR regulated inducible promoter; Erm^r	(128)
pSAS909	pYH3 vector with *yhcS* antisense RNA downstream of TetR ; Erm^r	(128)
pLH1	The integration vector pLL39 carrying Pspac-lacI segment, Tet^r	(194)
p312	pLH1 with *yhcSR* cloned downstream of the P*spac* promoter; Tet^r	This Study
pYhcSR	pYH4 shuttle vector with TetR regulated promoter for over-expression of *yhcSR*; Erm^r	This Study
pNreABC	pYH4 shuttle vector with TetR regulated promoter for over-expression of *nreABC*; Erm^r	This Study
pKOR1	*E.coli/ S.aureus* shuttle vector, permits lambda recombination and *ccdB* selection; temperature sensitive in *S. aureus*; Cm^r	(191)
pKOR1-Δ*yhcSR*	pKOR1 with flanking *yhcSR* upstream/downstream segments to mediate deletion of operon	This study
pKOR1-Δ*nreABC*	pKOR1 with flanking *nreABC* upstream/downstream segments to mediate deletion of operon	This study

Table 3-3 Oligonucleotide sequences

Primers	Sequence
YhcS For pLH1-NotI RBS	5’-GCGGCCGCAGGAGGTTTAAACTATGGAACAAAGG ACGCGAC-3’
YhcR-Rev pLH1-BamHI	5’-GGATCCCTAAATCAACTTATTTTCCATTGCATAAATTGC-3’
scv4	5’-ACCCAGTTTGTAATTCCAGGAG-3’
scv10	5’-TATACCTCGATGATGTGCATAC-3’
scv8	5’-GCACATAATTGCTCACAGCCA-3’
scv9	5’-GCTGATCTAACAATCCAATCCA-3’
attB1-yhcS-L-F-pKOR1	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGTACATGT ATGGCCAAACAACC-3’
yhcS-L-R-pKOR1	5’-ATAAACCTGCGCGATTAATGGATAA-3’
yhcR-R-F-pKOR1	5’-Phos-GGATAAAATTCCCCAAACAGAAAG-3’
attB2-yhcR-R-R-pKOR1	5’-TCACATAATAAGTCTGTTGCGTTATCTAC-3’
Yhc KO For	5’-CCTGGTCCAGTCGATGAATTG-3’
Yhc KO Rev	5’-CACTTGTTGGTGCATGGTTTG-3’
YhcS-RT	For 5'-GGGTCAGCCATCAAACGTATTTATTTAAC-3' Rev 5'-GAAATGAAAGACGTTGAGCCTCAGCA-3'
16S-RT	For 5'-CTGTGCACATCTTGACGGTA-3' Rev 5'-TCAGCGTCAGTTACAGACCA -3'
AttB1-pKOR1-nreKO-L-For	5’-GGGG ACAAGTTTGTACAAAAAAGCAGGCTGATACGCAA AAAGAACGTGGG-3’
pKOR1-nreKO-L-Rev	5’-CAAATCCTTTCACCTCTTATGCTTAC-3’
pKOR-nreKO-R-For	5’Phos-GTGAAAACAGTAGAAGCACATAAGAC-3’
AttB2-pKOR1-nreKO-R-Rev	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCGCGTTTAT AAACAGCAAGACTTA G-3’
NreABC-orf-PmeI-F	5’-GTTTAAACTATGACACCAGAAGCAATAATTGAAG-3’
NreABC-orf-AscI-R	For 5’-GGCGCGCCCTAAAACTCTAATAATTTCTTTTTC AATGCATATTC-3’
YhcSOE-for	For 5'-AAACTATGAACAAAGTAATATTAGTAG-3'
YhcROE-rev	For 5’-TTGGCGCGCCCTATTTTATAGGAATTGTGAATTG-3’

3.4. Analysis of essentiality and characterization of the *S. aureus yhcSR* two-component system.

3.4.1 Induction of *yhcSR* antisense RNA inhibits growth of *S. aureus* MSSA strain Newman and CA-MRSA strain 923.

It has previously been shown that induction of *yhcS* antisense RNA results in growth inhibition and suggests the *yhcSR* TCS is essential in a hospital-associated (HA)-MRSA strain, WCUH29 (100, 128). A recent publication suggested the essential nature of YhcSR was strain specific, as their methicillin-sensitive *S. aureus* (MSSA) strain Newman *yhcS* antisense RNA strain had no growth defect (129). To investigate this claim, an *yhcS* antisense RNA plasmid was introduced into *S. aureus* Newman and community-associated (CA)-MRSA *S. aureus* 923 (Fig. 3-1). Contrary to the other authors' results, *S. aureus* Newman was found to be extremely sensitive to induction of *yhcS* antisense RNA, with the *yhcS* antisense RNA strain Newman strain yielding poorer growth without induction of the *yhcS* antisense RNA compared to the empty vector control. Strain Newman's growth was severely inhibited with as little as 250 ng/µl of antisense RNA inducer, anhydrotetracycline (ATc) in TSB. The authors of the contradicting article did not provide evidence that their antisense RNA efficiently reduced *yhcS* mRNA or protein product, thus it is likely that the antisense RNA was ineffective and did not work as designed. It was previously demonstrated *yhcS* mRNA knockdown and YhcR protein loss occur during induction of *yhcS* antisense RNA with ATc (128). A similar growth defect was seen in *S. aureus* 923, but higher induction of the *yhcS* antisense RNA was needed to inhibit growth. The difference

in sensitivity to the *yhcS* antisense RNA between MSSA Newman and the MRSA strains may be due individual expression levels of *yhcSR* within each strain as well as inherent genetic differences via the absence of presence of additional mobile genetic elements that encode additional regulators which may influence *yhcSR* expression (195, 196).

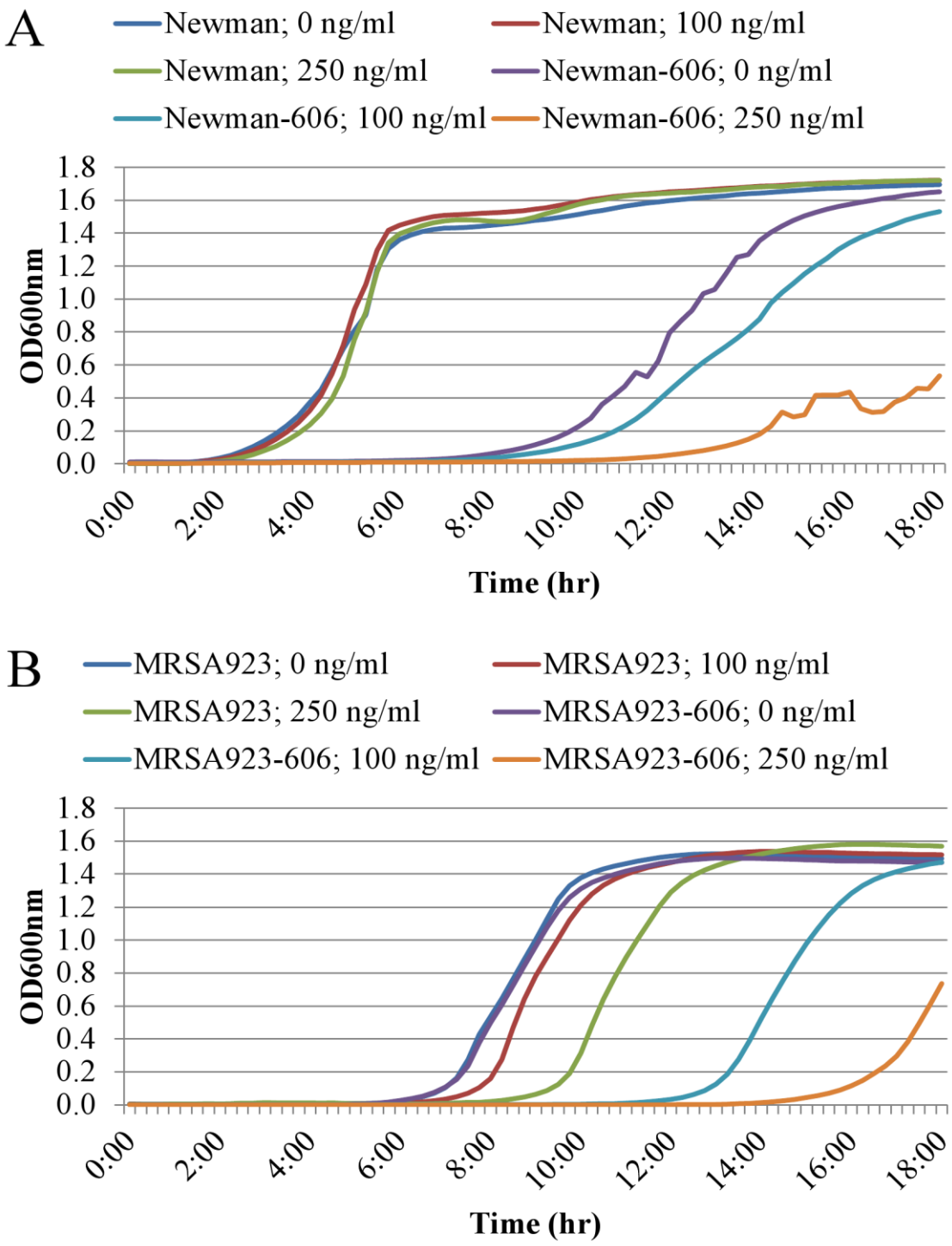

Figure 3-1 Growth of *S. aureus* Newman and *S. aureus* 923 with *yhcS* antisense RNA plasmid.
(A) Growth curves of the control Newman strain and *yhcS* antisense RNA strain, Newman-606 and **(B)** Growth curves of the MRSA923 control and *yhcS* antisense RNA strain, MRSA923-606, with various concentrations of inducer ATc. The growth curves represent one of three repeated experiments.

3.4.2 The *yhcSR* operon is essential for *S. aureus* WCUH29 aerobic and anaerobic growth.

Previously it was demonstrated deletion of the native *yhcSR* locus is only possible when *yhcSR* is heterologously expressed from another point on the chromosome in the highly mutagenized laboratory strain RN4200 (128). Using a method similarly used to confirm essentiality of the *walKR* (*yycFG*) TCS of *S. aureus* (197) an attempt to delete *yhcSR* was undertaken aerobically and anaerobically to confirm the essentiality of *yhcSR* during aerobic and anaerobic growth in the clinically relevant HA-MRSA strain WCUH29. Deletion of *yhcSR* only occurred in the presence of a heterologously expressed wild-type copy of *yhcSR* (placed in the Φ11 integration site) (Fig. 3-1A) (128, 190). Screening of bacteria grown in an anaerobic COY chamber produced similar results, deletion of *yhcSR* could only be obtained in the presence of a second heterologously expressed wild-type copy of *yhcSR* (Fig. 3-1B) indicating the essential nature of the *yhcSR* operon in *S. aureus* WCUH29.

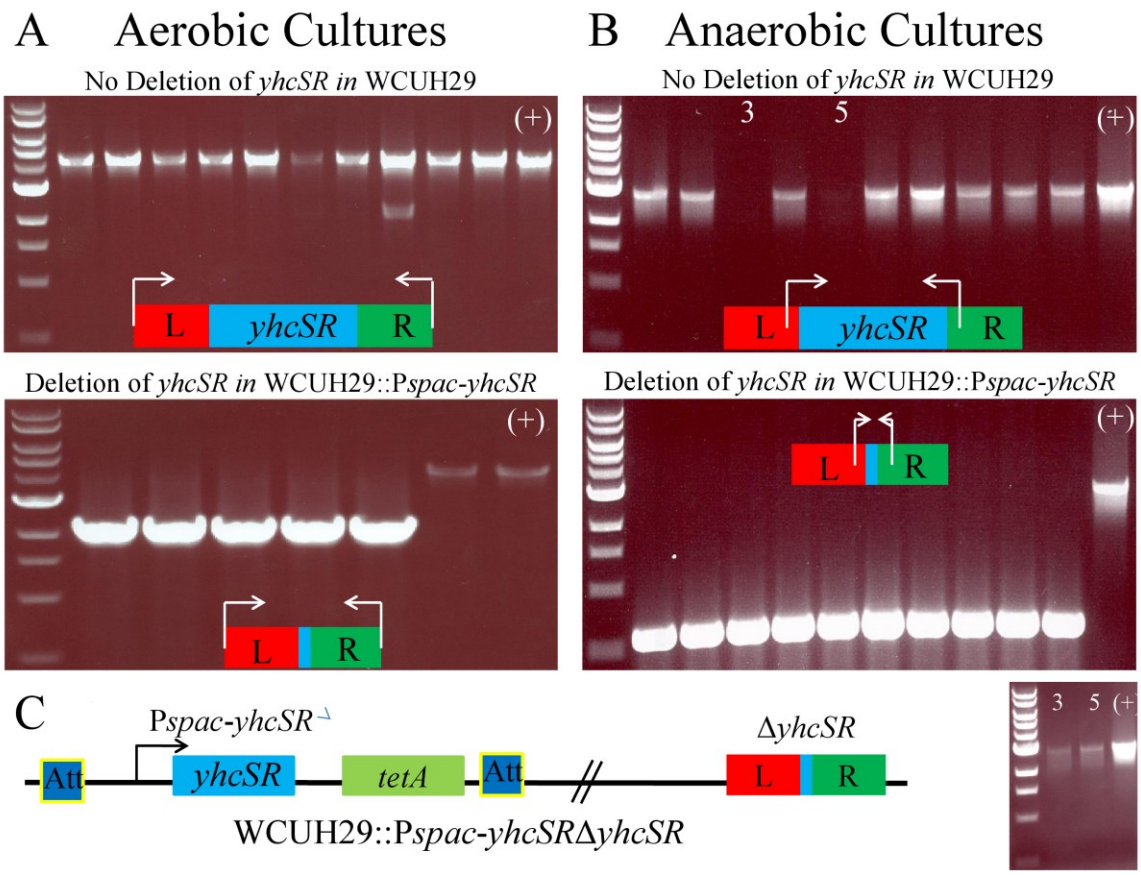

Figure 3-2. The native *yhcSR* operon can only be deleted when *yhcSR* is heterologously expressed.
In parallel, pKOR1-Δ*yhcSR* was electroporated into WCUH29 WT and WCUH29::P*spac-yhcSR* (JH213) and the pKOR1 deletion protocol was followed. Plates were incubated **(A)** aerobically or **(B)** anaerobically. **(C)** Outline of the completed WCUH29::P*spac-yhcSR*Δ*yhcSR* strain. White arrows indicate the placement of PCR primers to detect the presence or absence of the native *yhcSR* locus. Each lane represents a single PCR reaction of a randomly chosen colony. (+) represents wild-type WCUH29 genomic DNA used as a positive PCR control.

3.4.3 Transcriptional analysis of *yhcSR* expression during oxygen replete and deplete conditions.

Analysis to confirm the absolute essentiality of *yhcSR* indicated the operon was essential, but it did not provide information about when *yhcSR* is expressed during *in vitro* culturing. To determine when *yhcSR* was transcriptionally expressed and how anaerobiosis affected the transcription of the operon reporter bioluminescence production driven by the *yhcSR* promoter was monitored. A previously constructed P*yhcSR-luxABCDE* reporter plasmid, pCY106, and *S. aureus* WCUH29 strain, YJ106 (100) was utilized to determine the transcription of P*yhc* during growth phases and absence of oxygen. To produce micro-aerobic culture conditions, the TSB medium was autoclaved and cysteine was added to a concentration of 5.7 mM from a freshly prepared stock to scavenge free oxygen (198). As seen in Fig. 3-3, the growth rate of the semi-aerobic YJ106 culture is slower compared to the aerobic culture, but bioluminescence expression from the *yhcSR* promoter is almost 3-fold higher suggesting *yhcSR* is induced under anaerobic conditions. During aerobic growth, *yhcSR* transcription is maximally expressed in the early logarithmic phase of growth. In the semi-aerobic culture a similar, but amplified, expression pattern is seen with expression of *yhcSR* being sustained through, at least, the mid-logarithmic phase of growth. To confirm that *yhcSR* is expressed at a higher level when oxygen concentration is low or absent, qRT-PCR was used to compare the mRNA transcript level of *yhcS* in aerobically and anaerobically grown cultures of *S. aureus* WCUH29.

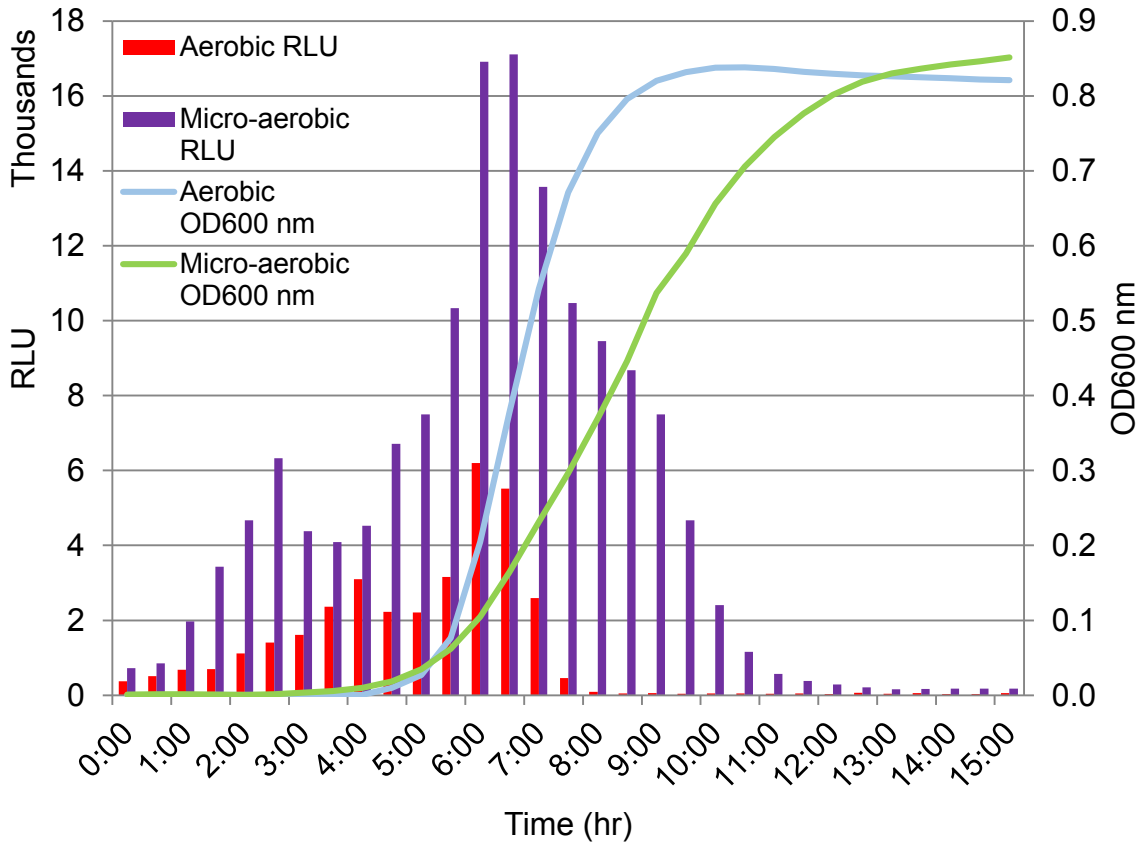

Figure 3-3 Analysis of transcriptional expression of the *yhcSR* operon using a P*yhc-luxABCDE* reporter fusion.
YJ106 in TSB containing appropriate antibiotics and in the absence (blue line, red columns) or presence of 5.7 mM cysteine (green line, purple columns). The bioluminescence signal and cell growth were monitored at 37ºC by measuring the light intensity and $OD600_{nm}$ with a BioTek Synergy II spectrophotometer every 30 min. The data represent the mean RLU and $OD600_{nm}$ of three independent cultures.

The qRT-PCR analysis revealed *yhcS* mRNA was 16-fold higher in the anaerobic culture compared to aerobic culture. Taken together, data show that *yhcSR* is expressed during aerobic and anaerobic growth of *S. aureus* and that transcriptional expression of *yhcSR* is higher when oxygen is absent from the culture.

3.4.4 Altering the expression level of y*hcSR* affects *S. aureus* cell size and morphology.

In an effort to characterize and understand the functional role YhcSR has within *S. aureus* it was investigated how altering the expression level of y*hcSR* affects cells size and morphology. Overnight cultures of induced controls, WSASJ909, and WYhcR were Gram-strained and cellular morphology was observed under oil immersion and 100X magnification. The Gram-stain revealed fairly uniform cocci in grape-like clusters in the control strains; a classic Gram-stain pattern of *S. aureus* (Fig. 3-4A and D). Intriguingly, depletion of *yhcSR* mRNA by *yhcS* antisense RNA produced heterogeneous cell morphology with the observation of overly large cells (Fig. 3-4B, black arrows). These cells had intact cell walls, as they retained crystal violet, but were 2- to 2.5-times larger in diameter than the control cells. Measuring the cell diameter of induced WJSAS909 cells revealed a large range in cell diameter, with a portion of the cells being similar in diameter to the controls, but a large portion of cells being dramatically larger than the control strain (Fig. 3-4C). Overproduction of YhcR produced fairly homogenous cellular morphology compared to the control. WYhcR cells were uniformly cocci in shape and clustered similarly to the control

cells. Visually, there was a noticeable increase in cell diameter in WYhcR cells compared to the control (Fig. 3-4D and E). This observation is supported by quantitative measurement of WYhcR cell diameter, which are on average 0.2 microns larger than the control cells (Fig. 3-4F).

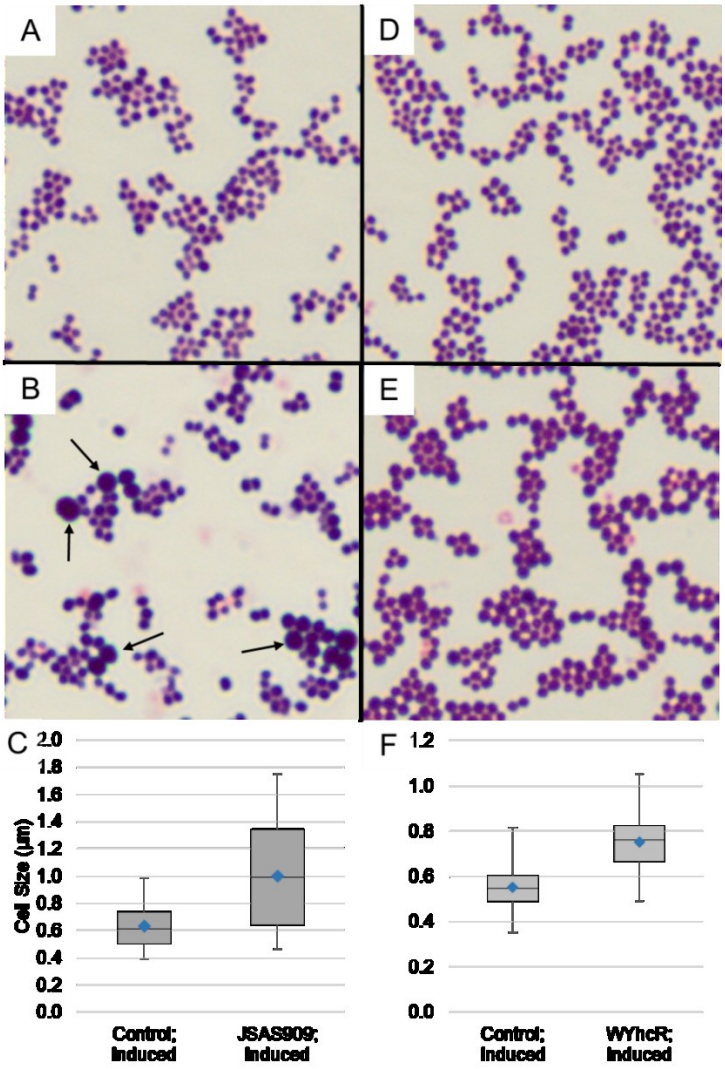

Figure 3-4 Altering the expression level of *yhcSR* affects *S. aureus* cell size and morphology. Gram strains of overnight cultures of **(A)** antisense RNA control strain and **(B)** *yhcS* antisense RNA strain, WJSAS909, were induced with 500 ng/ml of ATc. **(D)** Overproduction control strain and **(E)** YhcR overproduction strain, WYhcR, were induced with 250 ng/ml ATc. Each respective control and mutant strain was processed on the same glass slide. Images taken at 100X under oil immersion. Box and Whisker Plot of cell diameter for **(C)** antisense strains and **(F)** Overproduction strains measured in microns. At least 200 individual cells were measured for each strain.

3.4.5 Depletion of *yhcSR* decreases the rate of dissimilatory nitrate reduction in *S. aureus* WCUH29.

The NreBC TCS regulates the *nar* operon responsible for dissimilatory nitrate reduction in *S. aureus*. It was subsequently identified that YhcSR regulates both the *nar* and *nre* operons under anaerobic conditions by directly binding the respective upstream promoter sequences. Additionally, down-regulation of y*hcSR* by antisense RNA led to decreased *nar* promoter activity as established by a promoter-luciferase assay (100). To determine if the decreased *nar* expression resulted in a functional decrease in the amount of nitrate reduced to nitrite, the culture nitrite concentration of the CDM medium and growth of *S. aureus* was monitored over time. It was found that induction of *yhcS* antisense RNA led to a functional decrease in conversion of nitrate to nitrite by *S. aureus* when adjusted for optical density (Fig. 3-5A), likely due to decreased *nar* expression. Interestingly, overproduction of YhcSR in an *nre* deletion background did not restore nitrate reduction or enhance growth. Conversion of nitrate to nitrite was undetectable in the YhcSR complemented WCUH29Δ*nreABC* strain (Fig. 3-5B). The data suggest YhcSR is an enhancer of *nar* expression, and likely *nre* due to co-transcription of the *nar-nre* operons (127, 199), under anaerobic conditions and that NreBC is essential for nitrate reduction. As further evidence that YhcSR failed to complement the deletion of *nreABC*, the WCUH29Δ*nreABC*/pYhcSR strain grew at the same rate as the WCUH29Δ*nreABC*/pYH4 strain, which is limited to less energy productive fermentative growth.

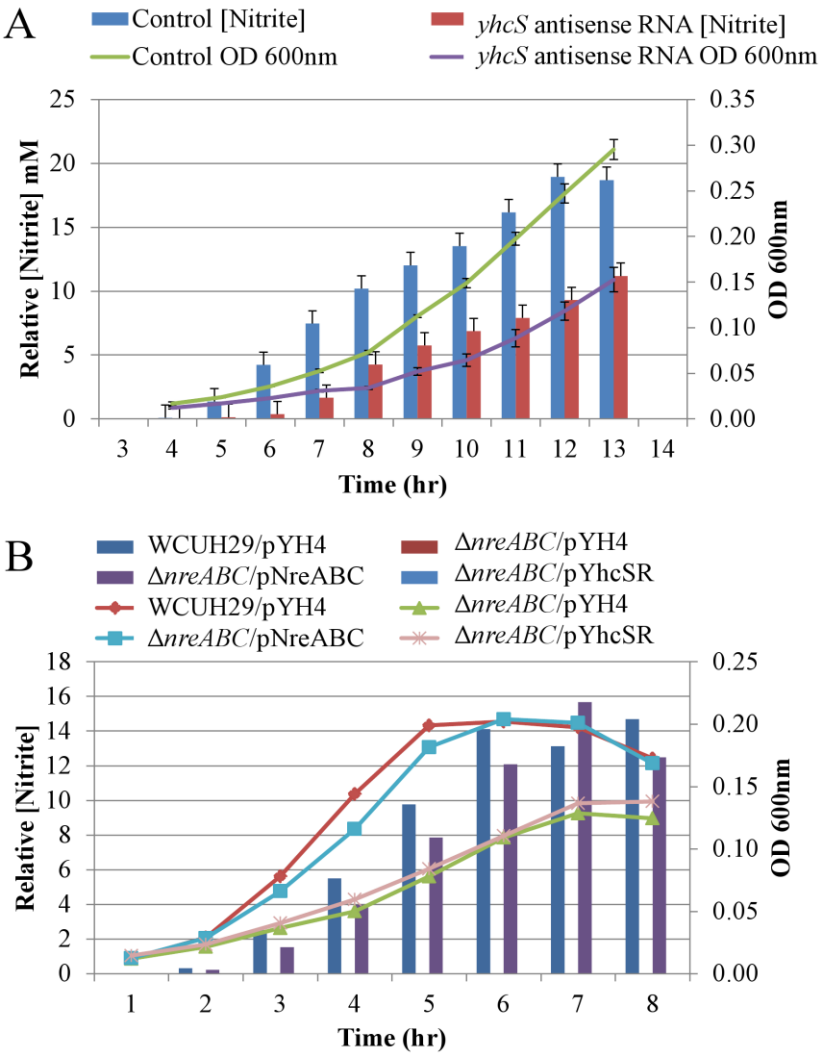

Figure 3-5 YhcSR is important for dissimilatory nitrate reduction but cannot complement the loss of *nreABC*.
(A) control (pCY406; green line; blue columns), *yhcS* antisense RNA strain (pCY606; purple line; red columns). Induction of *yhcS* antisense RNA produces a functional decrease in the amount of nitrate converted to nitrite in *S. aureus* cultures. Data represents the mean and standard deviation of 3 independent experiments. **(B)** Production of YhcSR in the WCUH29Δ*nreABC* background fails to complement nitrate reduction. Nitrate to nitrite conversion was not detected in WCUH29Δ*nreABC*/pYhcSR (represented by absence of blue columns) and growth complementation was not detected (compare pink line to blue line).

3.5 Discussion

In this study, it was determined that the *yhcSR* TCS is absolutely essential for *S. aureus* WCUH29 growth and expression *yhcS* antisense RNA in a CA-MRSA and MSSA strain inhibited growth in a similar manner as observed in WCUH29. It is concluded that the *yhcSR* TCS is extremely important for viability and likely to be essential for all strains of *S. aureus.* This is the first systematic analysis of *yhcSR* using an accepted merodiploid analysis for gene essentiality in *S. aureus* (200). Since the beginning of this work, there have been two articles published that dispute the essentiality of *yhcSR*. The first, published by Sun et al. (129) identified YhcS as an oxygen sensor and characterized the phosphorelay signal in relation to the absence or presence of oxygen. Sun et al. constructed a *bursa aurealis* transposon insertion mutant in YhcR, but failed to show that the transcription or translation of the gene was interrupted and a functional protein was not produced. Furthermore, a previous study investigating the role of TCSs in biofilm development also failed to recover a *yhcSR* deletion mutant (201). A second *bursa aurealis* transposon mutant library produced transposon insertions in *yhcS*, but failed to find a single insertional mutation in *yhcR* from a library of nearly 20,000 Tn insertion clones (202). This suggests *yhcS* may be dispensable for growth while the effector gene, *yhcR*, is the essential gene of the operon, but further investigation is necessary to determine the individual essentiality of each gene. Furthermore, Sun et al. claim that expression of *yhcS* antisense RNA does not inhibit growth of strain Newman, but again, evidence was not presented to show that the *yhcS* antisense RNA resulted in loss of the *yhcSR* mRNA or

inhibition of translation as was shown to occur for the previously constructed and published *yhcS* antisense RNA strain (128). As seen in Fig. 3-1, strain Newman is extremely sensitive to *yhcS* antisense RNA. While the Sun et al. paper does an excellent biochemical analysis of YhcSR, they fail to make a conclusive argument for non-essentiality of *yhcSR* in strain Newman.

A second, recent publication, reported that YhcSR modulates cell-wall synthesis genes (137). The concern with this report is not the claim that YhcSR modulates cell-wall synthesis, as they clearly show that YhcR binds the promoters of autolysis and cell-wall synthesis controlling genes. Additionally, the data presented in this chapter is supportive of this conclusion as it was found that altering the expression of *yhcSR* resulted in cells that appeared unable to divide, impacted cellular diameter and possibly cell-wall thickness. The disagreement is with their claim of constructing an *yhcSR ermC* replacement mutant. This is the first reported deletion of *yhcSR* in the absence of a second, ectopically expressed copy. While the authors note the controversy about *yhcSR* essentiality, they fail to include any information about the rate at which deletions in *yhcSR* were recovered or if they attempted to construct a deletion in a non-laboratory, clinically relevant strain of *S. aureus*. This is of particular interest because the *S. aureus* NCTC 8325 strain used in the report is a common laboratory strain and is known to have at least one major genetic defect, the absence of a functional *rsbU* gene which functions in activation of the stress induced alternative sigma factor, σ^B (203). It is possible that the construction of a true transposon mutant and gene replacement mutant were created and YhcSR

is not produced in the bacterium, making the gene not essential in these strains. So far though, supportive evidence of these claims is missing and it is quite possible during the construction of each of these mutants one or more suppressor mutations developed in the strains. Whole genome sequencing and comparative analysis between the parental and mutant strains is necessary to ensure this did not occur, followed by verification that the gene product of *yhcSR* is not made, or if it is translated, proteins are not functional in any manner.

As previously mentioned, YhcS has been shown to sense oxygen and directs YhcR to up-regulate genes necessary for anaerobic growth (129). In accordance with this observation and the previous publication indicating that YhcSR transcriptional regulates the *nar* and *nre* operons, depletion of YhcSR resulted in a functional defect in dissimilatory nitrate reduction resulting in a reduced growth rate in the presence of nitrate and YhcSR alone cannot complement for loss of NreABC, suggesting YhcSR is important for initiating transcription of *nre-nar* and subsequent prolonged production of NreABC is needed for nitrate respiration under anaerobic conditions.

It is concluded that the *yhcSR* TCS is essential for growth in all *S. aureus* strains based on the merodiploid analysis and *yhcS* antisense RNA data. It is very likely that YhcSR do regulate the genes identified in the other published studies; as results presented in this chapter and the following chapters are supportive and complementary to these claims. In the absence of any conclusive counter-data on the essentiality of *yhcSR,* investigations into novel inhibitors of the TCS should be undertaken in much the same way they have for the essential

WalKR TCS (113–115) as new antimicrobials to combat *S. aureus* infections are desperately needed.

The Essential *yhcSR* Two-Component Signal Transduction System Directly Regulates the *lac* and *opuCABCD* Operons of *Staphylococcus aureus*

Yan M, Hall JW, Yang J, Ji Y (2012) The Essential yhcSR Two-Component Signal Transduction System Directly Regulates the *lac* and *opuCABCD* Operons of *Staphylococcus aureus*. PLoS ONE 7(11): e50608. doi:10.1371/journal.pone.0050608

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Chapter 4 : The Essential *yhcSR* Two-Component Signal Transduction System Directly Regulates the *lacABCDE* and *opuCABCD* Operons of *Staphylococcus aureus*

4.1 Overview

Our previous studies suggested that the essential two-component signal transduction system, YhcSR, regulates the *opuCABCD* operon at the transcriptional level, and the *Pspac*-driven *opuCABCD* partially complements the lethal effects of *yhcS* antisense RNA expression in *Staphylococcus aureus*. However, the reason why *yhcSR* regulon is required for growth is still unclear. In this report, we present that the *lac* and *opuC* operons are directly transcriptionally regulated by YhcSR. Using real-time RT-PCR we showed that the down-regulation of *yhcSR* expression affected the transcription of *lacA* encoding galactose-6-phosphatase isomerase subunit LacA and *opuCA,* encoding a subunit of a glycine betaine/carnitine/choline ABC transporter. Promoter-*lux* reporter fusions further confirmed transcriptional regulation of *lac* by YhcSR. Gel shift assays revealed that YhcR binds to the promoter regions of the *lac* and *opuC* operons. Moreover, the *Pspac*-driven *lacABC* expression *in trans* was able to partially complement the lethal effect of induced *yhcS* antisense RNA. Likewise, *Pspac*-driven *opuCABCD* expression *in trans* enhanced growth of *S. aureus* in a high osmotic strength medium during induced *yhcS* antisense RNA expression. Taken together, the above data indicate that the *yhcSR* system directly regulates the expression of *lac* and *opuC* operons, which, in turn, may be partially associated with the essentiality of *yhcSR* in *S. aureus*. These results provide a new insight into the biological functions of the *yhcSR* TCS, a global regulatory system.

4.2 Introduction

The continuing increase of hospital- and community-associated methicillin resistant *Staphylococcus aureus* infections highlights an urgent need for the development of alternative potent antibacterial agents (141, 204, 205). The ability of this organism to resist current antibiotic therapies and cause infection is partially due to the coordinated regulation of gene expression allowing the bacteria to survive in different stress conditions. Two-component signal (TCS) transduction systems are important sensory units and allow microbial organisms to adapt to different niches, as well as play a significant role in pathogenesis and biofilm formation for various bacterial species(142, 206–208). Therefore, interrupting these critical signaling pathways may provide an alternative strategy for the development of novel classes of preventive and/or therapeutic antibacterial agents (195).

A typical two-component system is composed of a histidine kinase sensor and a cognate response regulator responsible for sensing and responding to environmental signals by regulating gene expression, respectively (207). In *S. aureus*, at least 16 different two-component signal systems have been identified (208), and two TCSs, *yycFG* and *yhcSR* (also known as *airSR*), are required for viability (128, 129, 197). The *yycFG* system, which has orthologs in *Bacillus subtilis* (209) and *Streptococcus pneumoniae* (210), is the first reported TCS regulatory system that is indispensable for cell viability in *S. aureus* (197). Analyses of conditional-lethal *yycFG* mutants in *B. subtilis* suggested that this system controls the *ftsAZ* operon that is involved in the process of cell-wall

division (211), as well as endopeptidase-type autolysins including YvcE and LytE (196). However, YycFG is not involved in the regulation of *ftsZ* expression in *S. pneumoniae* and *S. aureus*, but, modulates the expression of genes involved in cell wall metabolism in *S. aureus* (212), and an essential gene, *pcsB*, encoding a cysteine, histidine-dependent amidohydrolase /peptidase involved in cell wall biosynthesis in *S. pneumoniae* and *S. aureus* (212–216).

We have identified another TCS, *yhcSR*, which is also required for viability of *S. aureus* (128). However, the reason why *yhcSR* is required for viability and growth is unknown. In order to elucidate the biological functions of *yhcSR*, we examined the effect of *yhcSR* on gene expression using a microarray assay. Our preliminary microarray analysis suggested that the down-regulation of *yhcSR* expression affects the expression of genes associated with a variety of biological functions. Recently, we demonstrated that YhcSR positively regulates the *nreABC* and *narGHIJ* operons which are responsible for dissimilatory nitrate reduction under anaerobic growth conditions (100). We have also previously published data indicating regulation of the *opuCABCD* operon by YhcSR and showed that plasmid-borne expression of OpuCABCD partially complements the lethal effect of induced *yhcS* antisense RNA (193).

In this study, we employed quantitative RT-PCR, promoter-*lux* reporter fusions, and gel-shift technologies and demonstrated that the *yhcSR* system directly regulates the transcription of the *lac* operon encoding the structural genes for lactose and galactose metabolism, and the *opuCABCD* operon encoding a glycine betaine/carnitine/choline ABC transporter(217, 218).

Moreover, we found that that the regulation of the *lac* and *opuC* operons may partially contribute to the essentiality of the *yhcSR* regulon in *S. aureus*.

4.3 Material and methods

4.3.1 Bacterial strains, plasmids and growth media.

The bacterial strains and plasmids used in this study are listed in Table 4.1. The *S. aureus* cells were cultured in Trypticase soy broth (TSB) at 37˚C with shaking. *E. coli* strains were grown in Luria-Bertani (LB) medium. Transformants containing recombinant plasmids were selected on LB agar containing ampicillin (100μg/ml), chloramphenicol (50μg/ml), or erythromycin (300μg/ml) for *E. coli*, and TSA containing chloramphenicol (10μg/ml) or erythromycin (5μg/ml) for *S. aureus*. The isopropyl-β-D-thiogalactoside (IPTG) was added as 1mM at final concentration. Where noted, NaCl (1 M) and choline (1μM) were added to chemically defined medium (CDM).

4.3.2 RNA isolation, purification and qPCR.

Overnight cultures of *S. aureus* were inoculated 1:100 in TSB medium and grown to the mid-exponential (3 hr) phase of growth. Total RNA was purified from the above culture as described (192). Briefly, bacterial cells were harvested by centrifugation, and the RNA was isolated by the RNAPrep kit (Promega). Contaminating DNA was removed with a DNA-*free* kit (Ambion), and the RNA yield was determined spectrophotometrically at 260nm.

4.3.3 Quantitative real-time RT-PCR (qPCR) analysis.

In order to determine whether the down-regulation of *yhcSR* expression has any impact on the expression of several identified essential genes, we employed quantitative real-time reverse transcription (RT) PCR to compare the RNA levels, as described (166, 192). The first strand cDNA was synthesized using reverse transcriptase with the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen). For each RNA sample, we performed duplicate reactions of reverse transcription, as well as a control without reverse transcriptase, in order to determine the levels of DNA contamination. PCR reactions were set up in triplicate by using the SYBR Green PCR Master Mix (Strategene). Real-time sequence-specific detection and relative quantitation were performed with the Stratagene Mx3000P Real Time PCR System. Gene-specific primers were designed to yield ~100 bp of specific products (Table 4-2). Relative quantification of the product was calculated using the Comparative C_T method, as described for the Stratagene Mx3000P system. The housekeeping gene 16S rRNA was used as an endogenous control (166). All samples were analyzed in triplicate and normalized against 16S rRNA gene expression. The experiments were repeated at least three times. To determine whether the complementary effect was attributed to the over-expression of a *Pspac*-driven *lacABC* operon, we also utilized the qPCR as the above described.

4.3.4 Construction of promoter-*lux* reporter fusions.

In order to further confirm whether the *yhcSR* regulatory system transcriptionally regulates the expression of *lac* we created a promoter-*lux*

reporter fusion using the *yhcS* antisense expression vector, pCY606 as described and previously used to build an *opuC* promoter reporter (193). The promoter region of structural *lac* genes was amplified by PCR respectively using the primers (Sa1997proNotFor/ Sa1997proNotrev) listed in Table 4-2, digested with *NotI* and ligated upstream of the promoterless *luxABCDE* of pCY606, which was digested with the same enzymes. The resulting recombinant plasmids pMY1997 containing the promoter-*lux* reporter fusion was transformed into *E. coli* DH10B competent cells and correct orientation and DNA sequence was confirmed by PCR, restriction enzyme digestion, and DNA sequencing. The plasmid pMY1997 was purified and electroporated into *S. aureus* RN4220, and then into WCUH29, resulting in *S. aureus* strain, YJ1997. The *lux* expression was monitored during growth in TSB at 37°C with a Chiron luminometer. The relative light units (RLU) were calculated (bioluminescence intensity/optical density at 600_{nm}). Each experiment was repeated at least three times.

4.3.5 Cloning, expression and purification of YhcR-His tagged fusion protein in *Escherichia coli*.

In order to differentiate which identified genes are directly regulated by the *yhcSR* regulatory system, we cloned and purified a His-Tagged YhcR response regulator protein as described. The *yhcR* coding region was obtained by PCR from *S. aureus* and cloned into *Nde*I and *Xho*I sites of the *E. coli* expression vector pET24b. The recombinant plasmid (pET*yhcR*) was confirmed by PCR and DNA sequencing (data not shown) and transformed into *E. coli* strain BL21(DE3). The transformants were incubated until mid-log phase (OD600nm = ~0.6)

followed by induction of *yhcR* expression by adding IPTG (final concentration 1 mM). After two hours of incubation, cells were harvested and lysed by sonication. The overproduction of YhcR was viewed by SDS-PAGE followed by Coomassie Bright Blue staining (data not shown).

To purify the YhcR-His tagged protein a 500 ml culture of the BL21 (DE3) containing pET*yhcR* was induced, and the cell pellet was collected and lysed in Lysis Buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH8.0) by incubation at room temperature for 1 h with agitation. The supernatant was collected by centrifugation, applied to the Ni-NTA His-Binding Resin, and incubated for 30 min with shaking. The resin mixture was loaded onto the column and washed twice with washing buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH6.3). The YhcR-His protein was eluted with elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH5.6). Following Ni-NTA affinity purification the YhcR-His tag protein was further purified by size exclusion chromatography using Sephadex-50 (Sigma). The purified YhcR-His protein was analyzed by SDS-PAGE followed by Coomassie Bright Blue staining found to be the correct molecular weight (data not shown). The concentration of purified YhcR-His protein was determined by the Bradford method.

4.3.6 Protein phosphorylation *in vitro.*

For YhcR phosphorylation *in vitro*, total of 3 µg of protein was incubated in 30 µl of phosphorylation buffer [20 mM NaH_2PO_4 (pH8.0), 10 mM $MgCl_2$, 1 mM DTT, 32 mM acetyl phosphate] at 37˚C for 90 min as described (219).

4.3.7 Gel mobility shift DNA-binding assay.

To determine which identified gene(s) are directly regulated by YhcSR, we performed gel-shift assays. DNA fragments of the upstream DNA regions of *lacA* (*Plac,* 397 bp) and *opuCA* (*PopuC,* 312 bp) were obtained by PCR using the primers listed in Table 4-2. The amplified DNA fragments were purified and labeled with digoxigenin using the DIG GEL Shift Kits (Roche) according to the manufacture's protocol. The DNA-binding and electrophoresis were performed as described (168, 220). Briefly, the purified PCR products were labeled with digoxigenin (DIG) using terminal transferase (Roch). The labeled DNA fragments were further purified to remove the redundant DIG-ddUTP and salts. The interaction of YhcR-His with DNA was conducted in a 20 µl reaction mixture containing 0.2 pmol DIG-labeled DNA, 1 µg of poly-(dI–dC), 25 mM NaH_2PO_4 (PH 8.0), 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and different concentrations of YhcR-His protein (final concentrations of YhcR-His were 1, 2, 4, and 6 µM corresponding to 0.5, 1, 2, and 3 µg, respectively). Unlabeled DNA fragments of the promoter region as a specific competitor were added into the reaction with 100-fold excess to labeled probe. Bovine serum albumin (BSA) was used as a nonspecific binding control. The DNA binding reaction was initiated by the addition of YhcR-His and incubated at room temperature for 25 min. Samples were then loaded directly onto a 5% native polyacrylamide gel [acrylamide:bisacrylamide (29:1) in 0.5 x TBE buffer]. Samples were electrophoresed for 2 h at 4°C with 7V/cm, and the DNA samples were transferred to Nylon membrane via electro-blotting in 0.5 x TBE at 300 mA for 90

min at 4°C. After cross-linking of DNA fragments using UV light, the membrane was hybridized with anti-digoxigenin-AP antibody and exposed to X-ray film for 4 hours to achieve the desired signal.

4.3.8 Construction and characterization of *Pspac*-driven complementary strains.

In order to determine whether the modulation of *lac* by YhcSR is involved in the essentiality of YhcSR, we created *Pspac*-driven *lacABC* operon complement strains respectively within the *yhcS* antisense expression plasmid. Briefly, the 1905 bp PCR fragment of *lacABC* (sa1995-1997) was amplified using the primers Sa1997RBSfor/ Sa1995rev digested with *Not*I, then cloned into the same site of pMY107 as previously described (193). The resulting recombinant plasmid carrying the *lacABC* genes located downstream of the *Pspac* promoter region was obtained, confirmed by PCR and DNA sequencing, and correspondingly designated as pMY307. The pMY307 recombinant plasmid was electroporated into WCUH29 and resulted in *Pspac*-driven *lacABC* genes complement strains denoted as YJ307. To examine whether the *Pspac*-driven *lacABC* genes can complement the inhibitory growth effect of *yhcS* antisense RNA, we titrated the effect of induced *yhcS* antisense RNA on growth by kinetically monitoring the growth of the above complementary strains in TSB containing erythromycin (5μg/ml) and different concentrations of inducer, anhydrotetracycline (ATc) in a 96-well format using a SpectraMax plus Spectrophotometer (Molecular Devices) as previous described (128, 193). This experiment was repeated at least three times.

Table 4-1 Bacterial strains and plasmids

Strain	Description	Source
DH10B	*E. coli* strain used propagation and construction of plasmids	Invitrogen
BL21(DE3)	*E. coli* strain allows for overproduction and purification of heterologously proteins	Invitrogen
RN4220	*S. aureus* Laboratory strain: *rsbU*	(181)
WCUH29	Clinical human *S. aureus* isolate: *rsbU*$^+$	(221)
YJ2002	WCUH29 containing plasmid pYH3	
JSAS909	WCUH29 containing plasmid pSAS909 carrying *yhcS* antisense; Erm^r	(128)
YJ606	WCUH29 containing plasmid pCY606; Erm^r	(193)
YJ1997	WCUH29 containing plasmid pMY1997; Erm^r	This study
YJ107	WCUH29 containing plasmid pMY107; Erm^r	(193)
YJ207	WCUH29 containing plasmid pMY207; Erm^r	(193)
YJ307	WCUH29 containing plasmid pMY307; Erm^r	This study

Plasmids	Description	Source
pET24b	Vector for overproducing His-tagged proteins, Kan^r	Novagen
pyhcR-24b	pET24b derivative for overproduction of YhcR, Kan^r	This study
pYH3	Shuttle vector with a TetR regulated inducible promoter; Erm^r	(128)
pSAS909	pYH3 vector with *yhcS* antisense RNA downstream of TetR ; Erm^r	(128)
pCY606	Shuttle vector, derived from pSAS909, containing *luxABCDE* and *yhcS* antisense; Erm^r	(193)
pMY1997	Derived from pCY606, with sa1997 promoter - *luxABCDE* reporter fusion; Erm^r	This study
pMY107	Derived from pCY606, with *luxABCDE* replaced by *Pspac* promoter region; Erm^r	(193)
pMY207	Derived from pMY107, with sa2237operon under the *Pspac*; Erm^r	(193)
pMY307	Derived from pMY107, with sa1997operon under the *Pspac*; Erm^r	This study

Table 4-2 Oligonucleotide sequences

Primers	Sequence
yhcRforNdeI	5’-GGAATTCCATATGAACAAAGTAATATTAGTAG-3’
yhcRrevXhoI	5’-CCGCTCGAGAATCAACTTATTTTCCATTGC-3’
sa1997proNotFor	5’-TAGTGCGGCCGCATTAAAAGTATAACTGCATTG-3’
sa1997proNotRev	5’-TAGTGCGGCCGCTAATAAGACTCCTTTTTACTTT-3’
Sa2237prGSFor416bp	5’-TGCATTATTACAAAAATTCGAC-3’
Sa2237prGSRev416bp	5’-AACATAATCATTTCTCCTTCC-3’
Sa2237prGSFor312bp	5’-TATGAGTTATCTATTTAGTTGC-3’
Sa2237prGSFor210bp	5’-AGTAATCGGTAGAAATTCAAC-3’
Sa2237prGSFor172bp	5’-TACTGTTAAGTATTCACATTAC-3’
SA1997prGSfor	5’-TTAAAAGTATAACTGCATTG-3’
SA1997prGSrev	5’-CATCTGAACCAATAATAATC-3’
Pspacfor	5’-TCTAGAGCTGCCTGCCGCGTTTCGGT-3’
Pspacrev	5’-GCTTGAATTCCCGGGCGGCCGCCGCGGCCGGCC AATTGTTATCCGCTCACAATTC-3’
Sa1997RBSfor	5’-TAGTGCGGCCGCGGCCGGCCAGGAGGGAGTCTT ATT ATGGCGATTATTATTGG-3’
Sa1995rev	5’-TAGTGCGGCCGCGGCCGGCC TTACACCTCTAAAACTTCAATTTG-3’
SA2237RT	For 5’-CGTATCGGTGTCGTAAGAGCACT-3’ Rev 5’-GCACCACCTTACCTTCTGACAT-3’
SA1997RT	For 5’-TGGTGCAGGTAGCTTTATGGTTG-3’ Rev 5’-GCATATCTACGCGGATTTGGTGT-3’
SA0222RT	For 5’-AAGCAGTTAAAGAAGCAGACGAATCTTG-3’ Rev 5’-GTTGTGTTGTTTCTTCAGCTTTACCAG-3’
SA0959RT	For 5’-GGATACACCAGGACATGCAGACTT-3’ Rev 5’-CTACAACACCCTCTGGACGTGCT-3’
SA1044RT	For 5’-CCTAACACAAGACCGGTACCAGAT-3’ Rev 5’-CTTCACAGTGTGCTACGATGGCTT-3’
P2290	For 5’-TGTCGTCTTGAAATACGGCTGT-3’ Rev 5’-CTATATTGTTCGGTTTTTAAAAGCAATG-3’
SA1269RT	For 5’-GCCGTCAAGAGAGGCATTTGAAG-3’ Rev 5’-CAAGACCACCTGCTCCTACAACAA-3’
SA1271RT	For 5’-GATGCAACGATTGTGATGCCAG-3’ Rev 5’-ATTAGGCCACCTCCACCAACTG-3’

4.4 Results

4.4.1 Identification of essential genes regulated by the essential *yhcSR* system.

Using a *Pspac*-regulated *yhcSR* mutant and a TetR-regulated *yhcS* antisense RNA mutant, we have demonstrated that the down-regulation of *yhcSR* expression causes a lethal effect on bacterial growth (128). In order to elucidate the biological functions of the essential *yhcSR* system in *S. aureus*, we comprehensively examined the effect of conditional knockdown of *yhcSR* on gene expression using regulated antisense RNA technology with the combination of microarray analysis. Our preliminary microarray data showed that the down-regulation of *yhcS* expression differentially affected expression of various genes, including the *lac* operon encoding the structural genes for lactose and galactose metabolism (decreased expression two to five-fold) and *opuC* encoding a glycine betaine/carnitine/choline ABC transporter (decreased expression four to five-fold), and virulence factors (Table 4-3). To confirm the preliminary results, we first selected four down-regulated genes and five up-regulated genes including several essential genes and conducted qPCR analysis. For the control strain, YJ2002 (WCUH29 carrying pYH3, (221), the addition of inducer (250ng/ml of ATc) had no significant influence on the expression of the above selected 10 genes in both microarray and qPCR assays (data not shown). However, for the *yhcS* antisense RNA expression strain JAS909, the qPCR analysis showed that the down-regulation of *yhcSR* led to a 4-fold decrease of both *lacA* and *opuCA* expressions, which are consistent with the microarray data (Table 4-3).

Interestingly, we found that the down-regulated *yhcSR* expression significantly increased the transcription of several genes encoding virulence factors, including coagulase, fibronectin binding protein, and exotoxin, suggesting that the YhcSR system may function as a repressor of these virulence factor genes (Table 4-3), possibly through regulation of *agr* and *saeR* (129).

Table 4-3 qPCR and microarray data of gene expression in mid-log phase of growth, using the *yhcS* antisense RNA strain

Gene	Description	Fold Change	
		qPCR* (control)	Microarray
coa	staphylocoagulase	+4 (0)	5.59
set15	exotoxin 15	+4 (0)	2.5
SA0959	GTP-binding elongation factor homolog	+2 (0)	2.3
pyrC	dihydroorotase	+2 (0)	2.8
fnbB	fibronectin-binding protein homolog	+2 (0)	7.29
SA1269	Blt-like protein	-4 (0)	-33.4
SA1271	threonine dehydratase	-8 (0)	-51.67
lacA	galactose-6-phosphate isomerase	-4 (0)	-4.89
opuCA	Glycine/betaine/carnitine/choline-ABC transporter	-4 (0)	-4.39

qPCR* (control): The number inside parentheses is the fold change of specific genes in control strain YJ2002 with versus without ATc. – represents down-regulated gene expression during depletion of the *yhcSR* system; + represents up-regulated gene expression during depletion of the *yhcSR* system

4.4.2 Confirmation of transcriptional regulation of *lac* using a promoter-*lux* reporter fusion system.

To further confirm whether the *yhcSR* regulator transcriptionally regulates the expression of the *lac* genes, we created a promoter-*lux* reporter fusion system using the TetR-regulated *yhcS* antisense expression vector, pCY606. The promoter-*lux* reporter fusion strains (YJ1997) and the parental control strain (YJ606) were grown in TSB in the presence of the *yhcS* antisense RNA inducer ATc (200ng/ml) and in the absence of ATc, at 37°C with shaking. Bioluminescence intensity and optical density of the cultures were measured at different times of growth. No light signal was detected for the control strain YJ606 with and without inducer, suggesting no detectable leaky *luxABCDE* transcription (data not shown) and expression. However, for the *lac* promoter-*lux* fusion strains (YJ1997), the light intensity obviously decreased in the early log-phase of culture during the induction of *yhcS* antisense RNA expression with inducer ATc (Fig. 4-1). These results, in conjunction with our previous results on *opuC* (193), indicate that the YhcSR system transcriptionally regulates the expression of *lac* operon and *opuC* operons.

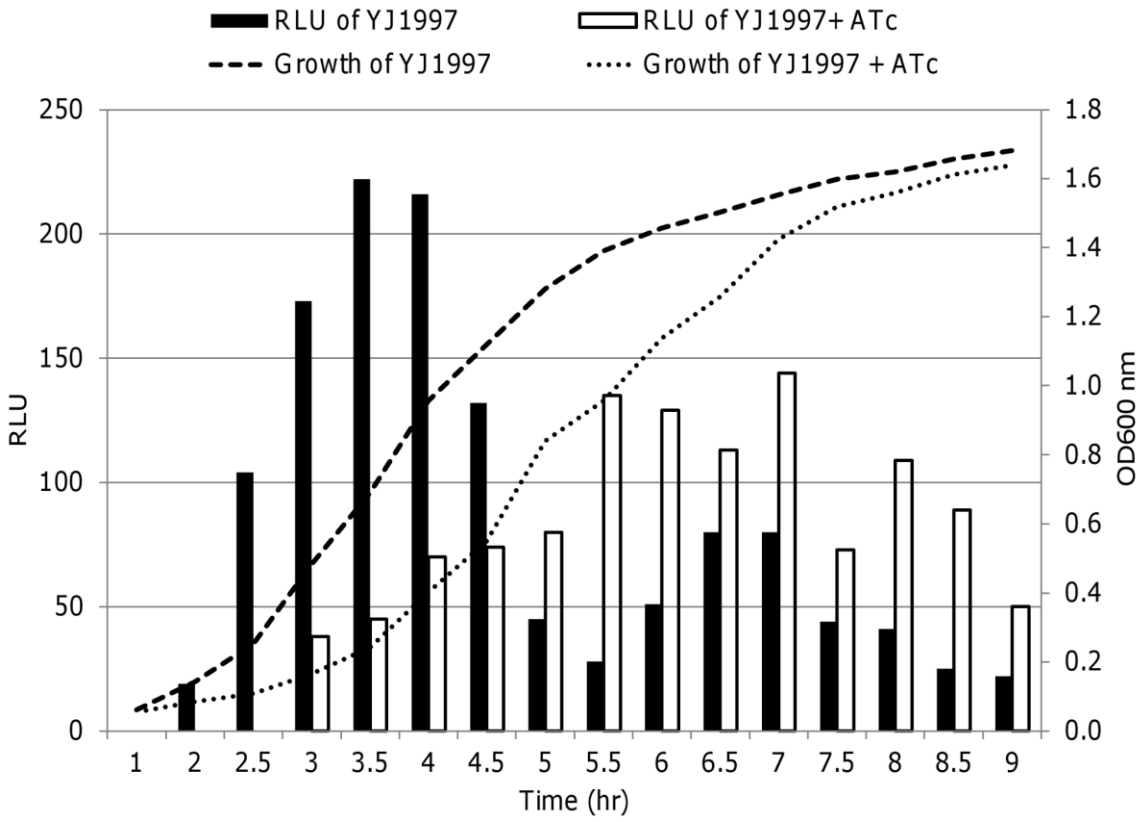

Figure 4-1 Analysis of transcriptional regulation of *lac* operon by the YhcSR system using promoter-*luxABCDE* reporter fusion.
The impact of the down-regulation of *yhcSR* on the expression of *lac* operon was determined by monitoring the bioluminescence intensity during growth. The overnight cultures of *S. aureus* strains were diluted to ~10^4 CFU/ml with TSB containing appropriate antibiotics and in the absence (solid dots) or presence of 200 ng/ml of inducer, ATc (solid dashes). Both bioluminescence signals and cell growth were monitored at 37°C by measuring the light intensity with a Chiron luminometer and optical density at 600 nm ($OD600_{nm}$) with a SpectraMax plus spectrophotometer every 30 min. To eliminate the effect of bacterial growth, the relative light units (RLU) were calculated (light intensity/$OD600_{nm}$) from triplicate readings at different times during growth.

4.4.3 YhcR directly interacts with the upstream promoter regions of *lacA* and *opuCA*.

To examine whether YhcR directly or indirectly regulates the expression of these essential genes, we conducted gel shift assays with the upstream regions of *lacA* and *opuCA*. Gel shift promoter probes containing the 300 to 400 bp upstream of the translational start site of the first structural gene of each operon were obtained by PCR and labeled with digoxigenin (DIG). Each gel shift assay consisted of a DNA probe-only control, the probe incubated with different concentrations of YhcR ranging from 1 to 6 μM, the probe plus 100-fold excess of unlabeled probe, and the probe with nonspecific protein BSA (2.3 μM).

The gel shift assays with the upstream region of *lacA* resulted in obvious bands shifted in a dose-dependent manner compared to the probe only and nonspecific proteins. Moreover, the addition of excess-unlabeled competitor apparently competed with the shifted band (Fig. 4-2A). Furthermore, two apparent shifted bands were revealed in the gel shift analysis with the upstream region of *opuCA* probe, suggesting the possible presence of multiple YhcR binding sites in the promoter sequence. The excess-unlabeled competitor successfully competed with the shifted bands (Fig. 4-2B). These results indicate that the YhcR response regulator may directly interact with the upstream regions of *lacA* and *opuCA* genes.

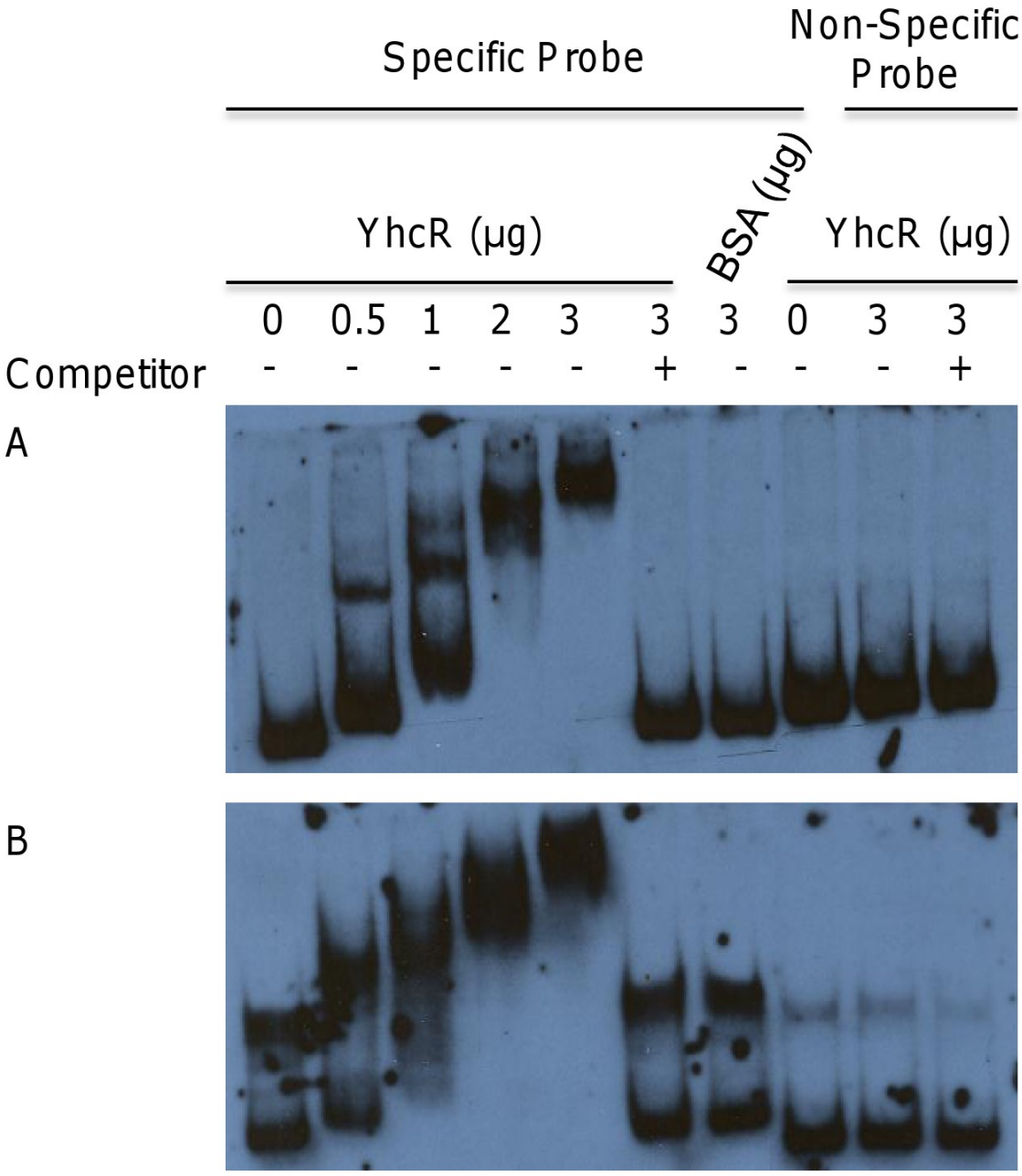

Figure 4-2 Gel-shift mobility analysis of genes regulated by YhcR. Different amounts of YhcR were incubated with each DIG-labeled promoter probes: P*lac* **(A)** and P*opuC* **(B)**. (–) represents without unlabeled specific competitor; (+) represents in the presence of 100-fold excess unlabeled specific competitor.

4.4.4 Expression of *lacABC* genes *in trans* partially complements the lethal effect of down-regulating *yhcSR* expression.

In order to determine whether the direct modulation of the *lac* operon is associated with the essentiality of *yhcSR* system, we conducted complementary experiments using a multi-copy plasmid. The *lacABC* genes were obtained by PCR using a high fidelity *pfx* DNA polymerase, and cloned downstream of *Pspac* promoter within the *yhcS* antisense RNA expression vector, pMY107 and labeled at pMY307. The recombinant plasmid was electroporated into the laboratory strain, RN4220, and subsequently, into the clinical isolate, WCUH29, labeled YJ307. The growth of the complementary strain YJ307 and control carrying parental plasmid DNA (YJ107) was kinetically monitored with increasing concentrations of inducer, ATc. Consistent with our previous findings (128), the induction of *yhcS* antisense expression with the inducer ATc dramatically inhibited the growth of the control strain (YJ107) in a dose-dependent manner (Fig. 4-3A). In contrast, the expression of the *lacABC* genes partially restored the growth of the *yhcS* antisense RNA expression strain and shortened the differential lag-phase of growth from five to two hours in the presence of 250, 500, and 750 ng/ml of inducer ATc (Fig. 4-3B). We previously observed similar results of partial complementation with the *opuC* operon (193).

To exclude the possibility that the above complementation may be attributed to the overproduction of the LacABC or OpuCABCD proteins in the multi-copy plasmid, we compared the growth between the control strain and each complementary strain in the absence of inducer. Without the induction of *yhcS*

antisense RNA expression, the complemented strain carrying the multi-copy plasmids of *lacABC* genes and the *opuC* operon showed a similar pattern of growth as the control strain showed (Fig. 4-3C).

To determine if the complemented growth effect of the *lacABC* genes and *opuC* operon on the *yhcS* antisense RNA lethal function resulted from the *Pspac*-driven *lacABC* genes and *opuC* operon over-expressions *in trans*, we performed qPCR using *lacA*, and *opuCA* specific primers (Table 4-2). Total RNA was isolated and purified from log-phase cultures, and cDNA was reverse transcribed. The transcriptional levels of *lacA* and *opuCA* in the complemented strains (YJ307 and YJ207) respectively increased 16- and 4-fold than those in the control strain (YJ107), suggesting the complemented growth effect seen in the assays was due to the *Pspac*-driven expression of the *lacABC* genes and *opuC* operon.

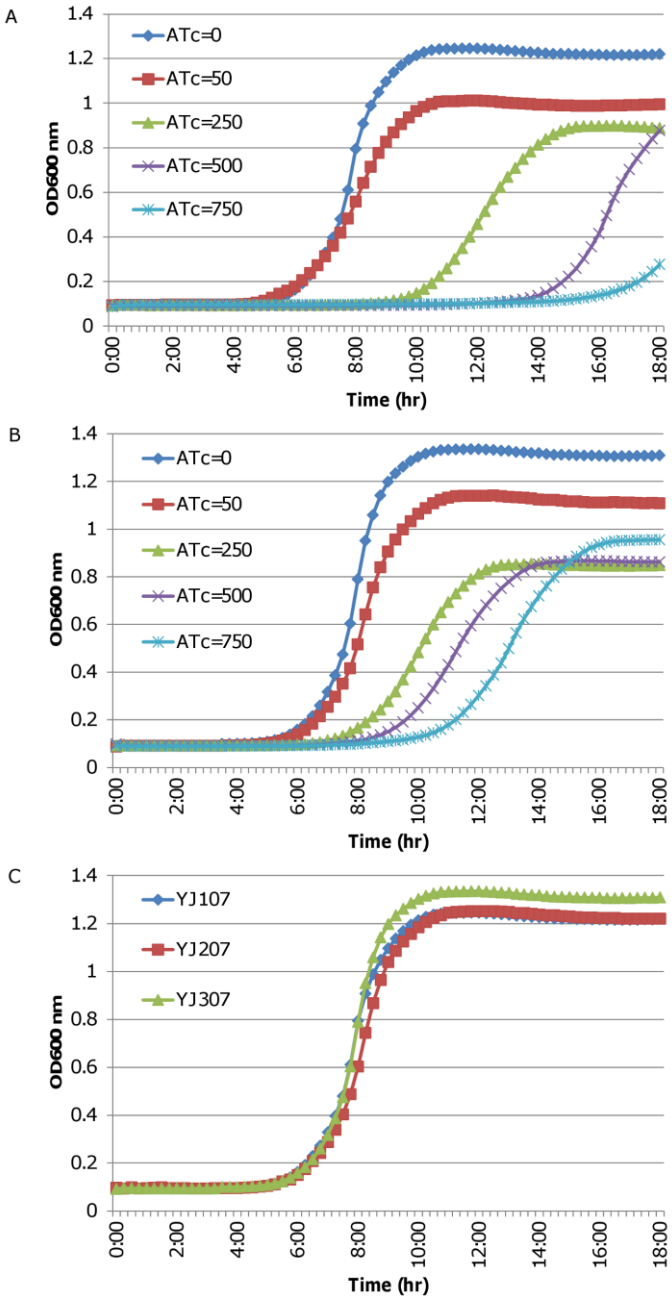

Figure 4-3 Complementation of induced *yhcS* antisense RNA by P*spac*-driven *lacABC* expression.

Growth curves of control strain, YJ107 **(A)**, *lacABC* complemented strain, YJ307 **(B)**, in TSB containing 5 µg/ml of erythromycin and various concentrations of inducer anhydrotetracycline, ATc (in nanograms/milliliter). **(C)** Represents the growth curves of the above strains in the absence of ATc. The overnight cultures of S. aureus strains were diluted to ~ 10^4 CFU/ml. Cell growth was monitored at 37ºC by measuring the optical density at 600 nm (OD_{600}) every 15 min, with 1 min of mixing before each reading in a SpectraMax plus spectrophotometer. The growth curves represent one of three repeated experiments.

4.4.5 Expression of o*puCABCD* genes *in trans* enhances growth in high osmolarity medium during *yhcS* antisense RNA induction.

In order to further to demonstrate the link between *yhcSR* regulation of the *opuC* operon we conducted complementation experiments using an *opuC* operon expressing multicopy plasmid and chemically defined medium in the absence or presence of NaCl and the compatible solute, choline. Choline, oxidized in the cell to glycine betaine, is one of several osmoprotectants that *S. aureus* can use to shield itself from high osmolarity environments (222, 223). Consistent with previous publications regarding *S. aureus* growth in high osmotic conditions (218, 223), the addition of NaCl to the CDM dramatically reduced the growth rate of the control strain YJ2002. The addition of choline to the CDM improved the growth of YJ2002. The presence of ATc had mild effect on the growth of *S. aureus* in all conditions, but the effect was equal across of the growth conditions for YJ2002 (Fig. 4-4A). The *yhcS* antisense RNA strain, YJ107 grew similarly to YJ2002 with ATc. As expected, induction of *yhcS* antisense RNA caused a growth defect in YJ107, this growth defect was further exacerbated with the addition of NaCl to the CDM. Adding choline to the NaCl-CDM allowed YJ107 growth to partially recover, similar to YJ2002 in the same medium (Fig. 4-4B). The presence of the P*spac*-driven *opuCABCD* genes in YJ207 resulted in *S. aureus* growing better in CDM containing NaCl and ATc, indicating protection of the cells from the high salt conditions. This protection may be mediated by OpuC transport of the minimal amount of L-proline in the CDM. The presence of the *opuCABCD* genes and the addition of choline to the NaCl-ATc-CDM further enhanced the growth of

YJ207 to the point that it grew as well as the YJ207 + ATc (Fig. 4-4C) This indicates that the P*spac*-driven expression of *opuCABCD* complemented the loss of the *yhcSR* regulated chromosomal *opuCABCD*.

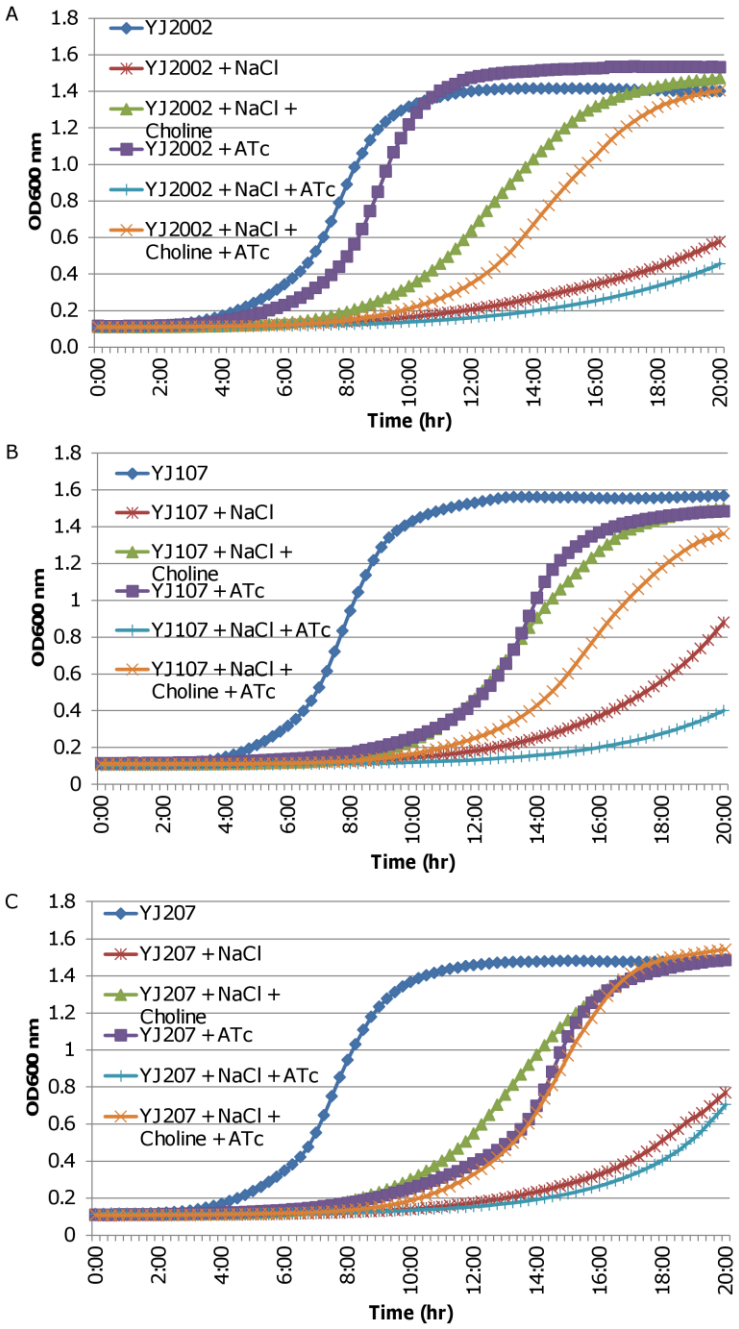

Figure 4-4 Complementation of induced *yhcS* antisense RNA by P*spac*-driven *opuCABCDE* in the presence of NaCl and choline.
Growth curves of control strain, YJ2002 **(A)**, *yhcSR* antisense RNA strain, YJ107 **(B)**, and *opuCABCD* complemented strain, YJ207 **(C)** in CDM with 750 ng/ul ATc, 1 M NaCl, and 1 µM choline chloride were added where indicated. The overnight cultures of *S. aureus* strains were diluted with CDM containing appropriate antibiotics and additives as indicated. Cell growth was monitored at 37ºC by measuring the optical density at 600 nm ($OD600_{nm}$) every 15 min, with 1 min of mixing before each reading in a BioTek Synergy 2 microplate reader. The growth curves represent one of three repeated experiments.

4.5 Discussion

Two-component signal transduction systems play important roles in the ability of bacteria to adapt to various environments by sensing alterations in their surroundings and by altering gene expression (207). With the availability of *S. aureus* genome sequences, at least 16 pairs of two-component signal transduction systems have been revealed (224). However, most of them have not been functionally explored. Previous work in our laboratory led to the identification of a novel essential two-component signal transduction system, *yhcSR,* in *S. aureus* (128) and demonstrated its role in regulating disimilatory nitrate reduction (100). In this study, we investigated two additional target genes that are directly regulated by the essential YhcSR system.

Our results demonstrate for the first time that the novel essential YhcSR system directly regulates the expression of *lac* and *opuC* operons (225, 226). Using microarray approaches, we and other investigators have successfully identified genes that are directly and/or indirectly regulated by different regulators including two-component signal regulatory systems, such as ArlRS (166), SaeRS (117), AgrA and SarA (227), and MgrA (228). In this study, we employed a similar approach to identify genes that are modulated by the YhcSR system. Our preliminary microarray data showed that the depletion of the YhcSR system apparently down-regulates the expression of *lac* and *opuC* operons, which were confirmed by semi-quantitative RT-PCR analysis. To further confirm whether YhcSR is able to regulate the expression of the *lac* operon at the transcriptional level, we performed promoter-*lux* reporter fusion assays. Consistent with the RT-

PCR data, the down-regulation of *yhcSR* expression obviously decreased the bioluminescence intensity of the *Plac-lux* promoter fusion. Taken together, and in conjunction with our previous publication (193) the results indicate that the essential *yhcSR* system transcriptionally regulates the *lac* and *opuC* operons in *S. aureus*. The different profiles of *lux* expression in the different promoter-*lux* reporter fusions during different stages of growth suggest that in the early log-phase YhcR controls *lac* transcription; in contrast, in the middle and late log-phases YhcR positively modulates *opuC* operon expression (193). This finding is not surprising since it has been found that the well-studied *agr* system differentially regulates the expression of both cell-wall associated proteins and exported toxins in different phases of cell growth (33).

In order to elucidate whether the above regulatory effects on the *lac* and *opuC* operons expression are mediated directly by YhcR or indirectly through other regulators, we employed gel shift assays. The upstream promoter regions of the *lac* and *opuC* operons bound to YhcR-His in a dose-dependent manner. We also found that the upstream promoter regions showed multiple shifted bands with a low concentration of YhcR, suggesting that these promoter regions may bind YhcR as a dimer or at multiple sites. This phenomenon has been observed with different regulators, including OmpR, SarA, and SrrA (220, 229, 230). These results indicate that YhcR positively mediates the expression of the *lac* and *opuC* operons by directly binding to their promoter regions.

In addition, unlike OmpR where binding affinity to its target DNA is increased by phosphorylation (229, 231), the phosphorylation of YhcR had no

obvious impact on our gel shift assays (data not shown), which is consistent with the report of SrrA (230). We cannot dismiss the possibility that the *in vitro* phosphorylation of YhcR in this study is not as effective as expected. It remains to be determined whether a phosphorylated YhcR has different binding sites and/or affinities in the promoter regions of *lac* and *opuC*.

To address whether the above transcriptional effects of YhcSR on *lac* expression are relevant to the biological function or essentiality of the *yhcSR* system, we conducted complementation experiments by introducing *Pspac*-driven *lacABC* genes in a multi-copy plasmid. The complementation of the *lacABC* dramatically curtailed the inhibitory effect of the induced *yhcS* antisense RNA. Biochemical evidence indicates YhcSR is an oxygen sensing two-component system (129), thus regulation of the *lac* operon by YhcSR links lactose fermentation to the absence of oxygen. The importation and ultimate fermentation of lactose provides an additional energy source during oxygen limited periods for growth.

A similar result of partial complementation was observed for *Pspac*-driven *opuC* operon (193). Additionally, we found that the down-regulation of *yhcSR* expression effectively inhibited bacterial growth in a high osmotic defined medium. Complementation with the *Pspac*-driven *opuCABCD* enhanced growth of the bacteria in the high osmotic medium and supplementation of the high osmotic medium with choline restore bacterial growth to that equal of the cells in CDM with ATc alone. This indicates full complementation by the *Pspac*-driven *opuCABCD* is occurring. Further complementation by the *Pspac*-driven *opuCABCD* is not seen,

nor expected, as the YhcSR TCS is a global regulator and likely controls other, as yet unidentified, essential genes. The data indicate that YhcSR is involved in the modulation of the transportation of the osmoprotectant choline through direct regulation of *opuC* operon expression. The transport of choline by OpuCABCD and their role in osmoprotection has been is well established (218, 232–234).

In *S. aureus*, OpuCA interacts with and influences the level of phosphorylated TRAP protein, a protein that protects DNA from oxidative stress (218, 235). The kinase activity of YhcS is influenced by oxidative stress (such as H_2O_2) (129) and the regulation of *opuC* by YhcSR is likely part of the overall stress response that occurs when *S. aureus* encounters stressful environmental conditions. The up-regulation of multiple stress response pathways likely allows the cell to better handle additional environmental insults.

Structural alignment indicates that YhcS and YhcR are more than 40% identical to YhcY and YhcZ in *B. subtilis*. However, unlike the YycFG system which is essential for *B. subtilis* and *S. aureus* (197, 209), YhcYZ is dispensable for *B. subtilis* growth (236). The YhcSR system may play different roles in *S. aureus* compared to the YhcHZ system in *B. subtilis*. In *B. subtilis*, only a limited set of genes are regulated by YhcHZ (237). In contrast, in *S. aureus* our preliminary microarray results showed that more than 80 genes are modulated by YhcSR (data not shown). This is also true for the YycFG system because YycF is involved in the regulation of the FtsAZ operon in *B. subtilis* (211), whereas there is no such evidence in *S. aureus* and *S. pneumoniae* (108, 212, 214–216, 224).

Interestingly, our preliminary microarray and qPCR analyses also indicate that the essential YhcSR system is probably a repressor of virulence factors because the down-regulation of *yhcSR* expression dramatically increased the expression of coagulase, fibronectin binding protein, and exotoxin (Table 3-3), possibly through regulation of the *agr* and *sae* operons (129). This suggests that the YhcSR system may function as a global regulator.

Chapter 5 : The YhcSR two-component system positively regulates the *sspABC* operon

5.1 Overview

To date, genes identified to be transcriptionally regulated by the YhcSR have been involved in energy production and cellular homeostasis of the cell. It is well accepted that the state of cellular metabolism impacts the expression of virulence factors in *S. aureus.* For this reason, experiments to determine if the YhcSR TCS is involved in *S. aureus* pathogenesis were conducted using antisense RNA and overproduction plasmids to create inducible mutants of this essential TCS. Likely due to growth inhibition, depletion of YhcSR resulted in decreased survival of *S. aureus* while the overproduction of YhcR promoted survival of *S. aureus* in human whole blood. This enhanced survival is linked to the direct transcriptional regulation of the *sspABC* operon, encoding V8 protease (SspA), staphopain B (SspB) and staphostatin B (SspC). SspA and SspB are known virulence factors which proteolytically digest opsonins and inhibit the function of professional phagocytes by cleavage of receptors. Deletion of *sspAB* partially eliminated the YhcR-mediated enhanced survival in human blood. This is the first evidence linking the TCS to pathogenesis of *S. aureus*.

5.2 Introduction

Staphylococcus aureus accounted for approximately 20% of bloodstream infections in the U.S. (238). The bacteria gain access to the bloodstream commonly from the result of puncture wounds of the skin (239–241), surgical site infections, or insertion of central venous lines and catheters (238, 242). Once *S. aureus* enters the bloodstream the bacteria have the ability to enter almost any site of the human body (243). *S. aureus* bloodstream infections often lead to septic shock and endocarditis (144). Bacteremia accounted for 75% of invasive *S. aureus* infections identified by the Active Bacterial Core Surveillance program, a nationwide observation program of federal and state health officials. Septic shock and endocarditis accounted for an additional 10% of invasive infections (7).

The pathogenicity of *S. aureus* partially relies on the coordinately-regulated expression of virulence factors that allow the bacterium to evade the host immune system and/or promote survival during infection. Similar to other bacterial pathogens (244–249), *S. aureus* has evolved a series of regulatory effectors (11, 213, 245, 250, 251) which allow the organism to sense and to adapt to changing environmental stimuli and survive within a particular niche by modulating specific cellular responses and virulence gene expression. Sixteen two-component systems are encoded in the core *S. aureus* genome, with many of them influencing the expression of virulence factors (92, 95–97, 119, 123, 201, 252–254). Some of these TCSs link cellular metabolism and virulence factor expression to the availability of extracellular nutrients, such as KdpDE and

HssRS systems that sense extracellular K^+ and heme, respectively (98, 250). Analysis of YhcSR to date has shown the two-component system to be a sensor of oxygen (129) that modulates the expression of pathways responsible for dissimilatory nitrate reduction (100), cellular osmotic balance (193) and alternative sugar catabolism pathways (99). Importantly, the YhcSR TCS is essential for aerobic and anaerobic growth of *S. aureus* as described in chapter 1 (128, 130).

The mechanisms by which *S. aureus* survives and subverts the vertebrate immune system have been studied for many decades. These published studies identified many *S. aureus* produced immune suppression factors and that circulating neutrophils and monocytes are key innate cellular components to combat infection by *S. aureus* (255–260). Data presented in this chapter are the first evidence that indicate the YhcSR TCS transcriptionally activates expression of the *sspABC* operon, encoding V8 protease (*sspA,* serine endopeptidase), staphopain B (*sspB,* cysteine endopeptidase) and staphostatin B (*sspC*, inhibitor of Staphopain B). These proteases have been linked to a wide variety of innate immune system suppression pathways by their ability to degrade complement components (139), induce vascular leakage and promote extracellular matrix structural damage (261, 262). In addition, the proteases inhibit neutrophil chemotaxis and induce apoptosis of neutrophils or engulfment of neutrophils by macrophages (263, 264).

Overproduction of YhcR resulted in enhanced survival of *S. aureus* in human blood. Furthermore, the culture supernatants of the YhcR over-producing

S. aureus strain inhibited opsonin mediated phagocytosis. The enhanced survival of YhcR overproducing *S. aureus* is partially the result of direct transcriptional up-regulation of the *sspABC* operon which led to increased production of SspA and SspB, consequently opsonophagocytic clearance of the bacteria was likely inhibited in the blood survival assay.

5.3 Materials and methods

5.3.1 Bacterial strains, plasmids, and growth media.

The bacterial strains and plasmids used in this study are listed in Table 5-1. The *S. aureus* cells were cultured in trypticase soy broth (TSB) at 37˚C with shaking. *E. coli* strains were grown in Luria-Bertani (LB) broth. Transformants containing recombinant plasmids were selected on LB agar containing ampicillin (100 µg/ml), kanamycin (50 µg/ml), or erythromycin (300 µg/ml) for *E. coli*, and trypticase soy agar (TSA) containing chloramphenicol (10 µg/ml), tetracycline (5 µg/ml), and/or erythromycin (5 µg/ml) for *S. aureus*.

5.3.2 Blood survival assay.

Strains were cultured in TSB with appropriate antibiotics. Inducer ATc was added when indicated to overnight cultures. Following 18 hours of culturing, the bacteria were washed twice in sterile PBS and suspended to an OD of 0.14 using a Behring photometer in PBS. Fresh venous human whole blood was collected using heparin containing Vacutainer tubes (BD) from outwardly healthy adult donors. The blood was then immediately used in the assay. Approximately $5x10^6$ CFU in 50 µl of PBS were added to 450 µl of blood per microcentrifuge tube with

appropriate antibiotics and ATc, where indicated. Microcentrifuge tubes were capped and placed in a rotisserie incubator and incubated at 37°C with end-over-end mixing. At indicated time points a 20 µl sample was removed from each sample, serially diluted, and plated on TSA to determine the surviving CFU count for each sample. The percentage of surviving bacteria was calculated as $CFU_{time\ point}/CFU_{initial\ input}*100$.

5.3.3 Gene deletion protocol.

Deletion of *sspB* and *sspAB* was carried out following the pKOR1 allelic exchange protocol as described (191) and primers in listed in Table 5-2. Plasmid pJB38 is a modified version of pKOR1 (265) and pJB38-*sspAB* was kindly provided by Alex Horswill (266). All deletions were confirmed by diagnostic PCR.

5.3.4 Cloning, expression and purification of a YhcR-His tagged fusion protein in *Escherichia coli*.

The purification of YhcR-His was carried as described using the previously constructed pET*yhcR* plasmid described in section 4.3.3 (99). The only modification to the protocol was the use of Pro-Lyse™ Bacterial Lysis Buffer (Lamda Biotech) to lyse the *E. coli*.

5.3.5 SDS-PAGE analysis of exported proteins, mass spectrometry peptide-protein identification, and western blotting.

The culture supernatants were collected from the overnight cultures of *S. aureus* strain grown in TSB medium with 5 µg/ml of erythromycin and 250 ng/ml inducer ATc. Bacterial cells were pelleted by centrifugation at 3900 x g. The culture supernatants were then passed through a 0.2 µm syringe filter to remove

all bacterial cells. The exported proteins were precipitated from an equal volume of supernatant using ethanol as described (157). The exported protein profiles were detected by a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue staining. Five prominent overproduced protein bands (JH1-JH5) were cut from the gel and in-gel digested (267). Samples were submitted to the University of Minnesota, College of Biological Sciences Mass Spectrometry Core where they were processed for peptide identification by mass spectrometry. Western blotting for SspB was conducted as described previously (117) using a SspB antibody kindly provided by Alex Horswill (266) and an alkaline phosphatase conjugated anti-chicken secondary antibody (Sigma). Overnight cultures grew to similar $OD600_{nm}$ values and equal volume of precipitated protein from each culture supernatant was loaded rather than equal protein concentration so that differences in protein concentration could be observed.

5.3.6 Zymography analysis.

A 12% resolving SDS-PAGE gel with 0.1% final gelatin concentration was used for zymography analysis. Each sample was mixed with protein solubilisation buffer (5X, 50% glycerol, 10% (w/v) SDS, and 0.5 M Tris-HCl, pH 6.8) and incubated at room temperature for 30 minutes.

Each sample was loaded into the wells of the gelatin SDS-PAGE and electrophoresed at a constant 50V for 3 hours. After electrophoresis, the gel was placed in a plastic wash container and 1X SDS removal buffer (2.5% Triton X-100, 5 mM $MgCl_2$, 25 mM Tris-Cl, pH 7.5) was added. The gel was gently

washed in the SDS removal buffer for a total of 60 minutes at room temperature. The SDS removal buffer was replaced after 30 minutes with fresh removal buffer and then rinsed gently with DI water. Development buffer (0.1% Triton X-100, 5 mm $MgCl_2$, 25 mM Tris-Cl, and pH 7.5) was added until it covered the gel and the container was incubated at 37°C for 24 hours. After the development period, the development buffer was removed and the gel was rinsed gently with DI water. Stain buffer (50% DI water, 35% MetOH, 15% Glacial Acetic Acid, 0.25% Coomassie Blue R-250) was added to cover the gel in the container. The gel was incubated until it was no longer visible in the stain buffer. Stain buffer was removed and the gel was rinsed gently with DI water. Fixing buffer (2% Glacial Acetic Acid, 98% DI water) was added to the container until it covered the gel and incubated at room temperature for 24 hours.

5.3.7 Analysis of transcriptional regulation using a promoter-*lux* reporter fusion system.

To investigate if the *yhcSR* regulator transcriptionally regulates the expression of the *sspABC, agrBDCA, saePQRS* operons, promoter-*lux* reporter fusion plasmids were created. The *agr* promoter-*lux* reporter was provided by Philip Hill (117) and this plasmid served as the backbone for construction of the *sae* and *ssp* promoter-reporters. The upstream *sae* and *ssp* promoter regions were PCR amplified with primers listed in Table 5-2, digested with *Eco*RI and *Xma*I (NEB). The pCY1006 plasmid was digested with the same enzymes to remove the *agr* promoter fragment and the plasmid backbone was purified by agarose gel electrophoresis. Each PCR fragment was ligated into the gel purified

*Eco*RI/*Xma*I digested pCY1006 backbone with T4 DNA ligase (Promega). Re-constructed *sae*- (pCY306) and *ssp*-*lux* promoter reporters were confirmed by diagnostic PCR using the promoter specific forward primer and a plasmid specific *luxA* reverse primer listed Table 5-2. Plasmids were purified from *E. coli* DC10B and electroporated into the WCUH29-based strains as indicated in Table 5-1. The promoter-*lux* reporter fusion strains were grown in TSB in the absence of ATc, at 37°C with shaking overnight. Strains were diluted 1:300 and incubated without or with 25 ng/ml of inducer ATc. Bioluminescence intensity and optical density of the cultures were measured at different times of growth. The Relative Light Units (RLU) were calculated by dividing the bioluminescence reading by the $OD600_{nm}$ reading ($^{lum}/_{OD600nm}$) at each time point. Each experiment was repeated at least three times.

5.3.8 Electrophoretic mobility shift assay.

The same *ssp* promoter primers listed in Table 5-2 used for the luciferase reporter were ordered with a 5'-biotin tag. The 5'-biotin labeled promoter fragment was obtained by high-fidelity PCR. A total of 300 µl of PCR reaction was prepared and processed and loaded into a 2% agarose gel. The sample was electrophoresed, stained with ethidium bromide and the promoter fragment was removed from the gel and purified using a NucleoSpin Gel Clean-up kit (Macherey-Nagel). The purified probe was subjected to electrophoresis in a 5% native Tris-borate EDTA (TBE) PAGE gel for further purification. The fragment was cut from the gel and re-purified according to the NucleoSpin Gel Clean-up kit

protocol. Lastly, a sample of the purified probe was electrophoresed on a 1.2% agarose gel to verify the size and purity of the biotin labeled P*ssp* probe.

The Electrophoretic mobility shift sssay (EMSA) was performed essentially as described in the manufacturer's protocol. The LightShift Chemiluminescent EMSA Kit (Thermo Scientific) was used to perform the assay. All samples contained 1X LightShift Binding Buffer, 50 ng/µl Poly (dI•dC), and 2.5% glycerol. The labeled probe, YhcR-His, non-labeled specific probe, BSA, and non-specific non-labeled probe were all added to concentrations as outlined in the Fig. 5-12 and ultrapure water was added so that all reaction volumes totaled 20 µl. The reactions were incubated at room temperature for 20 minutes followed by addition of 5 µl of 5X loading buffer to each reaction. 20 µl of each reaction were loaded into the wells of a pre-run 8% TBE native polyacrylamide gel and electrophoresed at 75V for three hours at 4°C. The samples were transferred to nylon membrane and processed as outlined in the manufacturer's protocol. BioMax Light Film (Kodak) was used to detect the chemiluminescent reaction.

5.3.9 Construction of overproduction plasmids.

Gene ORFs were obtained using Q5 high-fidelity polymerase (NEB) PCR using the primers (YhcROE-for/YhcROE-rev; sspB-For/sspB-Rev-AscI; sspABC-For/sspABC-Rev-AscI) in Table 5-2. Purified PCR fragments were digested with *Asc*I. The pYH4 plasmid carrying the TetR regulated, anhydrotetracycline (ATc) inducible promoter was digested with *Pme*I and *Asc*I. Digested PCR fragments were ligated into the digested pYH4 plasmid with T4 DNA ligase (Promega) and confirmed by diagnostic PCR using the Tetfor/TTrev primer pair listed in Table 5-

2 that are specific for regions upstream and downstream of the TetR regulated promoter.

5.3.10 HL-60 opsonophagocytic killing assay.

HL-60 pluripotent cells were differentiated to granulocytic cells and cultured for 5 days as described (268). The basic assay consisted of approximately 1,000 CFU of *S. aureus* WCUH29 placed in duplicate of a 96 well microtiter plate. Twenty and 10 µl of human serum and complement from 3-4 week of white rabbits (Life Technologies), respectively, were added to each well. Lastly, 4×10^5 differentiated HL-60 granulocytes were added to each well to initiate the assay. The plates were incubated for 60 minutes at 37°C and with a concentration of 5% level of CO_2. Each well was mixed gently and 10 µl of sample from each well was drop plated in triplicate on TSA plates to determine surviving CFU. The percent survival was calculated as the number of surviving CFU/number of input CFU multiplied by 100, (CFU_f/CFU_i*100). The experiment was repeated at least 4 times. To determine the effect of *S. aureus* strains exported proteins on the activity of serum antibodies and complement, induced cultures were grown in TSB with appropriate antibiotics and 250 ng/ml of inducer ATc overnight at 37°C with shaking. The following day the bacterial cells were pelleted and the TSB culture supernatant was filter sterilized with a 0.2 µm syringe filter. Twenty five milliliters of each culture supernatant, along with sterile TSB as a vehicle control, were concentrated 50-fold using a Millipore Centrifugal Protein Concentrator with a 10 kD nominal molecular weight limit. Before the HL-60 phagocytic assay, each concentrated culture supernatant and TSB was mixed

1:1 with the human serum or rabbit complement, incubated at 37°C for 30 minutes, and then placed on ice. In the assay, 40 and 20 µl of the serum mixture and complement mixture, respectively, were added to each well. Additional buffer was added to the complement mixture wells so all wells were of equal 100 µl volume.

Table 5-1 Bacterial strains

Strain	Description	Source
DC10B	Dam$^-$ *E. coli* which allows for direct electroporation of purified plasmid DNA into wild-type *S. aureus*	(269)
BL21(DE3)	*E. coli* used for protein expression; IPTG inducible; Cm^r and Kan^r	Invitrogen
RN4220	Laboratory *S. aureus* strain (RsbU$^-$)	(181)
WCUH29	Human clinical MRSA isolate	NCIMB40771
JE2	Plasmid cured derivative of LAC strain, community-acquired MRSA isolate	(202)
923	Community-acquired MRSA isolate	
WCUH29/pYH3	WCUH29 antisense control strain with empty pYH3; Erm^r	(128)
JSAS909	WCUH29 with pYJY909; Erm^r	(128)
WCUH29/pYH4	WCUH29 protein overproduction control with empty pYH4; Erm^r	This Study
WYhcR	WCUH29 with pYH4-*yhcR*; Erm^r	This Study
JE2/pYH4	JE2 with pYH4; Erm^r	This Study
JE2/pYhcR	JE2 with pYH4-*yhcR*; Erm^r	This Study
923/pYH4	923 with pYH4; Erm^r	This Study
923/pYhcR	923 with pYH4-*yhcR*; Erm^r	This Study
NE1787	JE2 transposon mutant, *srtA::erm*; erm^r	NARSA
JH1787	NE1787 with *erm* gene removed from transposon; *srtA* mutant	This Study
JH1787/pYH4	JH1787 with pYH4; Erm^r	This Study
JH1787/pYhcR	JH1787 with pYH4-*yhcR;* Erm^r	This Study
WSspB	WCUH29 with pYH4-*sspB*; Erm^r	This Study
WCUH29/pYH4/ P*ssp-lux*	WCUH29/pYH4 with P*ssp-lux*; Erm^r	This Study
WYhcR/P*ssp-lux*	WYhcR with P*ssp-lux*; Erm^r	This Study
WYhcR/pCY1006	WYhcR with pCY1006; Erm^r, Cm^r	This Study
WYhcR/P*sae-lux*	WYhcR with pCY306; Erm^r, Cm^r	This Study
WCUH29*saeS*	WCUH29 transposon mutant, *saeS::tc,* Tc^r (371)	(117)
WCUH29*agrA*	WCUH29 transposon mutant, *agrA::tc,* Tc^r	(117)
WCUH29*saeS*/ pYH4/ P*ssp-lux*	371 with pYH4 and P*ssp-lux*; Erm^r, Cm^r	This Study
WCUH29*saeS*/ pYhcR/P*ssp-lux*	371 with pYH4-yhcR and P*ssp-lux*; Erm^r, Cm^r	This Study
WCUH29*agrA*/ pYH4/ P*ssp-lux*	WCUH29*agrA* with pYH4 and P*ssp-lux*; Erm^r, Cm^r	This Study
WCUH29*agrA*/ pYhcR/P*ssp-lux*	WCUH29*agrA* with pYH4-yhcR and P*ssp-lux*; Erm^r, Cm^r	This Study
JH114	WCUH29 with in-frame deletion of *sspAB*	This Study
JH214	WCUH29Δ*sspAB* with pYH4; Erm^r	This Study
JH314	WCUH29Δ*sspAB* with pYhcR; Erm^r	This Study
JH414	WCUH29Δ*sspAB* with pSspABC; Erm^r	This Study

Table 5-2 Plasmids

Plasmids	Description	Source
pCY1006	Shuttle vector carrying *agr* promoter-*gfp-lux* reporter, derives from pSB2019; Cm^r, Amp^r	(117)
pET*yhcR*	pET24b based for production of YhcR in *E.coli* BL21(DE3)	(99)
pYH3	Shuttle vector with a TetR regulated inducible promoter; Erm^r	(128)
pSAS909	pYH3 with *yhcS* antisense downstream of TetR promoter; Amp^r, Erm^r	(128)
pYH4	pYH3 with Amp^r removed; Erm^r	(270)
pYhcR	*yhcR* cloned downstream of pYH4 TetR promoter for overproduction; Erm^r	This Study
pTnT	Allelic exchange plasmid; allows for removal of *erm* gene in NE1787	(265)
pSspB	*sspB* cloned downstream of pYH4 TetR promoter for overproduction; Erm^r	This Study
P*ssp-lux*	*sspABC* promoter cloned upstream of promoterless *luxABCDE*; derived from pCY1006; Cm^r, Amp^r	This Study
pCY306	*Sae* promoter cloned upstream of promoterless *luxABCDE*; derived from pCY1006; Cm^r, Amp^r	This Study
pKOR1	Temp. sensitive inducible allelic exchange plasmid for *S. aureus*; Cm^r	(191)
pKOR1-*sspB*	pKOR1 with in-frame *sspB* upstream/downstream deletion region	This Study
pJB38-*sspAB*	pJB38 with in-frame *sspAB* upstream/downstream deletion region	(266)
pSspABC	*sspABC* operon cloned downstream of pYH4 TetR promoter for overproduction; Erm^r	This Study

Table 5-3 Oligonucleotide sequences

Primers	Sequence
srtA-For	5'-ATGAAAAAATGGACAAATCGATTAATGAC-3'
srtA-Rev	5'-TTATTTGACTTCTGTAGCTACAAAG-3'
ssp Pro For EcoRI	5'-TAGCGAATTCGATATGTTGAACTGGACGTCGTGAAC-3'
ssp Pro Rev XmaI	5'-TTCCCGGGCTAAAAACCTCCAAAAAATTTATTTACAAGTTAAATATAACAC-3'
sspB-For	5'-AGGAGGTTTAAACTATGAATAGTTCATATAAATCTAGAGTATTCAATATTATAAGC-3'
sspB-Rev-AscI	5'-TT*GGCGCGCC*TTAGTAACCTATCATTGAACCATACCAG-3'
sspABC-For	5'-AGGAGGTTTAAACTATGAAAGGTAAATTTTTAAAAGTTAGTTCTTTATTCG-3'
sspABC-Rev-AscI	5'-TT GGCGCGCCTTATACTAAGCGCTCATAAACGATTGG-3'
YhcROE-for	5'-AAACTATGGAACAAAGGACGCGAC-3'
YhcROE-rev	5'-TTGGCGCGCCCTATTTTATAGGAATTGTGAATTG-3'
sspB ORF-L-F-pKOR1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCGTTATCATCAAAAGCTATGGAC-3'
sspB ORF-L-R-pKOR1	5'-CATATAATACCCTCCATTTAAATCAAAATATTTTGC-3'
sspB ORF-R-F-pKOR1	5'-Phos-GATTATAACTGGTATGGTTCAATGATAGGTTAC-3'
sspB ORF-R-R-pKOR1	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAT TTGATGCAGGTTATGGTTCAGG
pJB38-ssp For	5'-GAATATGATATTAAGTCACTTGCGTCG-3'
pJB38-ssp Rev	5'-GCTTATGAAATGGATGTTTTAAAAGAAGGTATG-3'

5.4 Results

5.4.1 Perturbation of *yhcSR* expression alters the survival of *Staphylococcus aureus* in human blood.

It is important to validate the *in vivo* essentiality of any gene as some genes found to be essential *in vitro* may not be essential *in vivo* (271, 272). The essentiality of *yhcSR* makes a direct comparison of a wild-type strain and mutant impossible. As with our previous TSB medium studies, a *yhcS* antisense RNA and an a overproduction plasmid were used to examine the effects of YhcSR on survival in human whole blood.

Survival of the *yhcS* antisense RNA strain (JSAS909) in human blood was assayed as an initial step to determine the importance of *yhcSR* for survival in the host. Using a defined volume of blood allowed for the efficient and sufficient concentration of antisense RNA inducer, ATc to be added to the assay. As seen in Fig. 5-1A, as a percentage of the initial inoculum, depletion of YhcSR by *yhcS* antisense RNA (128) resulted in significantly decreased survival in the first hour of incubation in human blood and continued for the second hour of incubation. Similar numbers of colony forming units (CFUs) survive in the controls and uninduced *yhcS* antisense RNA strain whereas fewer CFUs survived in the blood for the induced *yhcS* antisense RNA strain throughout the experiment (Fig. 5-1B). The data suggest *yhcSR* are important for survival in blood and perhaps essential for survival in the broader host environment.

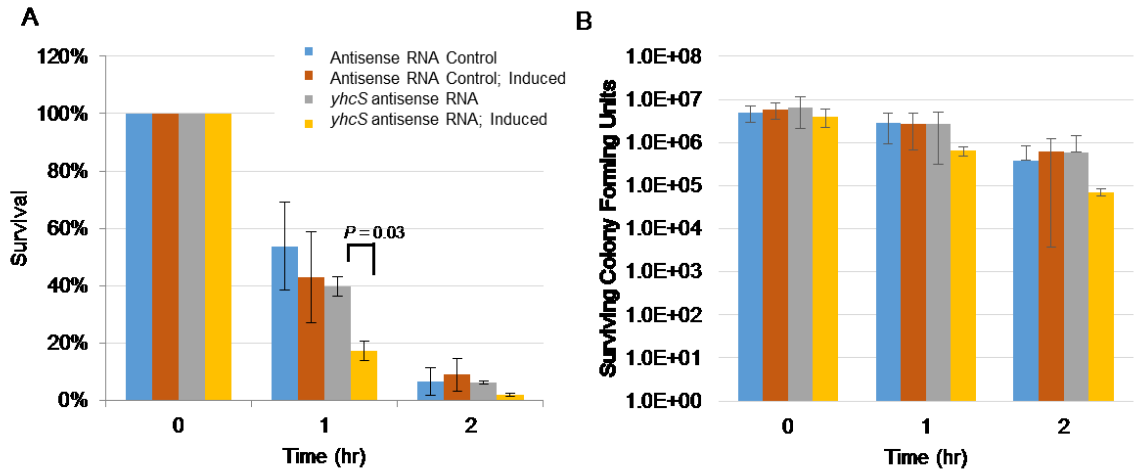

Figure 5-1 *yhcSR* are important for survival in human blood. (A) Percent survival of the *S. aureus yhcS* antisense strain (JSAS909) in human blood during ATc induction. Percent survival = CFU_t/CFU_i*100. Data represents the mean and standard deviation of four experiments. Statistical analysis conducted with Student's T-Test; p = ≤0.05. **(B)** CFU counts of each strain. Cultures of *S. aureus* strains were cultured overnight with or without the inducer ATc (500 ng/ml) and the following day diluted and inoculated into 500 ml of fresh blood with erythromycin and with or without the inducer ATc (500 ng/ml).

The depletion of YhcSR in *S. aureus* appeared to decrease the survival of the bacteria in human blood. To further investigate the importance of YhcSR for survival in blood an YhcR overproduction strain (WYhcR, Fig. 5-2) was used to determine if the overproduction of the response regulator could promote survival in blood.

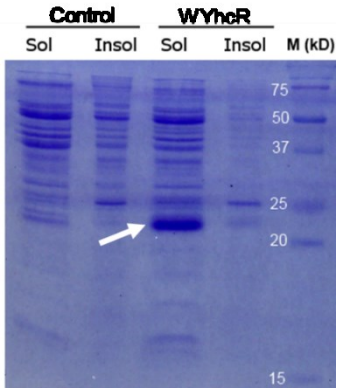

Figure 5-2 Induction of YhcR in *S. aureus* WCUH29. Cultures were induced overnight with 250 ng/ml of inducer ATc. The next day they were adjusted to an equal OD_{600nm}. The bacteria were incubated with lysostaphin to weaken the cell wall and then lysed with Pro-Lyse lysis solution. The insoluble portion was pelleted and soluble supernatant transferred to a fresh tube. Each fraction was subjected to SDS-PAGE. YhcR (White arrow) is induced and found in the soluble (cytoplasmic) portion of the bacteria

As seen in Fig. 5-3A, overproduction of YhcR promotes survival of *S. aureus* over the course of the three hour experiment as measured as a percentage of the initial inoculum. It was later determined that the overproduction of YhcR from the episomal plasmid increased the overall bacterial cell size, thus when cultures of each strain were adjusted to the same $OD600_{nm}$ the CFU/ml of the induced WYhcR strain was found to be somewhat lower than that of the controls (see section 3.4.5) so that in the first hour the total CFU survival appeared less than the controls (Fig 5-3B), but by percentage the survival is equal to the controls. By hour two of the experiment, there were twice as many surviving induced WYhcR CFU as the controls, further supporting the notion that YhcSR promotes survival of *S. aureus* in blood.

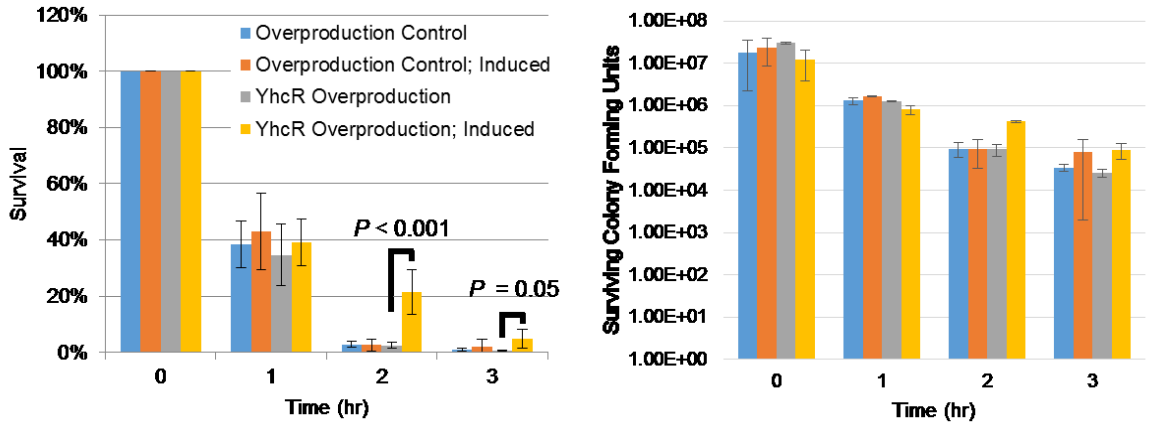

Figure 5-3 Overproduction of YhcR promotes survival of HA-MRSA *S. aureus* in human blood. (A) Percent survival of the *S. aureus* YhcR overproduction strain (WYhcR) in human blood during ATc induction. Data represents the mean and standard deviation of four experiments. Statistical analysis conducted by Student's T-Test; p = ≤0.05 (**B)** CFU counts of each strain. Cultures of *S. aureus* strains were cultured overnight with or without the inducer ATc (250 ng/ml) and the following day diluted and inoculated into 500 ml of fresh blood with erythromycin and with or without the inducer ATc (250 ng/ml).

To determine if the enhanced survival of *S. aureus* during YhcR overproduction was applicable to other genetic backgrounds of *S. aureus*, the pYH4 and pYhcR plasmids were transformed into community-acquired methicillin resistant *S. aureus* (CA-MRSA) strains, JE2 and 923. During the initial tests with WCUH29, no difference in percent survival between the control strain with or without inducer ATc and the uninduced WYhcR strain was observed. For this reason, the control and YhcR overproduction strain for JE2 and 923 were only assayed with inducer ATc. Furthermore, I found that, in general, only a small percentage of the WCUH29 cells survived beyond two hours, thus survival after 30 minutes to two hours after inoculation for the CA-MRSA and future assays was analyzed. Fig. 5-4 shows that overproduction of YhcR enhanced the survival of strains JE2 (Fig. 5-4A) and 923 (Fig. 5-4B) in blood. The data indicated

overproduction of YhcR enhanced survival in blood across several genetic backgrounds of *S. aureus* and suggested YhcSR contributes to *S. aureus* pathogenesis and survival in the host.

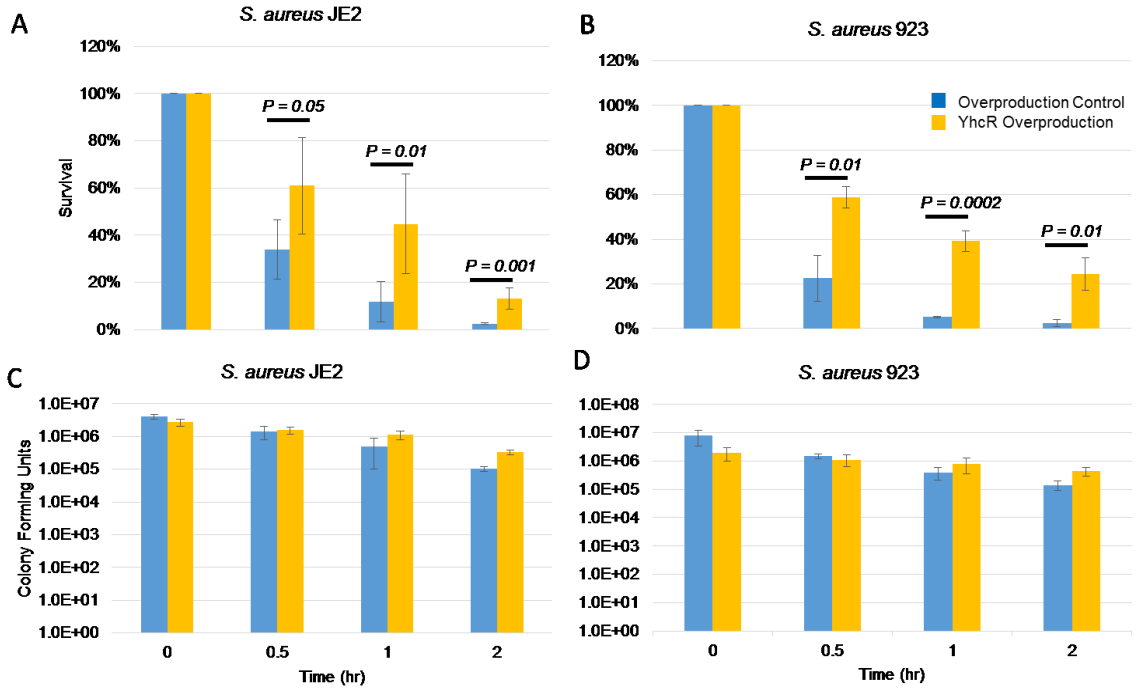

Figure 5-4 Overproduction of YhcR contributes to survival of CA-MRSA in human blood. Percent survival of the CA-MRSA *S. aureus* **(A)** JE2 and **(B)** 923 during YhcR overproduction. Data represents the mean and standard deviation of at least three experiments. Statistical analysis conducted by Student's T-Test; p = ≤0.05. CFU counts of each strain, **(C)** JE2 and **(D)** 923. Cultures of *S. aureus* strains were cultured overnight with inducer ATc (250 ng/ml) and the following day diluted and inoculated into 500 ml of fresh blood with erythromycin and with inducer ATc (250 ng/ml) and incubated at 37°C.

5.4.2 Sortase A attached cell-wall proteins likely do not contribute to enhanced survival in human blood during YhcR overproduction.

Some virulence factors of *S. aureus* are cell surface-associated proteins collectively termed, "microbial surface components recognizing adhesive matrix molecules" or MSCRAMMs, that mediate binding to a multitude of host factors as

well as promote intracellular adhesion (273). This group of proteins include the immunoglobulin binding proteins, protein A and Sbi, and the intracellular adhesion and fibrinogen-binding proteins, Efb and FnbA/B (274, 275), and the platelet aggregation factors ClfA/B (143). This group of proteins mediate a multitude of functions involved in tissue attachments, complement and cellular inhibition.

Many of these proteins are synthesized as a longer precursor protein with an N-terminal signal sequence that directs the protein into the sorting pathway. Protein sorting is mediated by the sortase pathway which recognizes a C-terminal LPXTG motif. Sortase A (*srtA*) cleaves the peptide bond between threonine and glycine and subsequently attaches the processed protein to the pentaglycine cross-bridge of newly synthesized peptidoglycan (276–280).

To determine if the factor(s) that promote bacterial survival during YhcR overproduction are Sortase A mediated cell-wall associated virulence factors, a *srtA* transposon mutant, NE1787, was obtained from the Network on Antimicrobial Resistance of *S. aureus* (NARSA) (202, 281). The transposon used for gene disruption carries an erythromycin resistance cassette which is incompatible with the erythromycin cassette carrying pYhcR expression plasmid. The pTnT plasmid was obtained from NARSA and the protocol that allowed for the removal of the erythromycin gene was followed. The rest of the transposon was left in place for continued gene disruption (265). Several colonies were screened by PCR for removal of the *erm* gene (Fig. 5-5A). Two colonies (#11 and #13) were identified to have lost the *erm* gene. Streaking colony #11 to TSA

without and with erythromycin showed that the strain was sensitive to erythromycin when compared to NE1787 and was labeled strain JH1787 (Fig 5-5B). To verify that the *srtA* gene was inactivated in the JH1787 strain, the protein content of different fractions of the parental wild-type JE2 strain, *srtA::erm* (NE1787), and *srtA::tn* (JH1787) strains (Fig. 5-5D) were separated by SDS-PAGE. Protein samples were transferred to a nitrocellulose membrane. An IgG secondary antibody was used for immunoblot detection of the *S. aureus* produced non-specific IgG binding protein, protein A, a sortase A cell-wall attached protein (282, 283). As seen in Fig. 5-5E, the immunoblot detects expression of protein A in all strains as seen in the lanes for the whole-cell analysis. Protein A can be cleaved from the bacterial cell wall by staphopain B and shed to the surround environment (138) and importantly protein A is not detected in the culture supernatant of JH1787 (or NE1787). Most convincing, protein A is not detected in the JH1787 cell-wall fraction, while it is readily detected in the cell-wall fraction of the parental JE2 strain (Fig 5-5E). The data highly suggest that the *srtA* gene is inactivated in JH1787 (and NE1787) and would suggest that sortase A attached cell-wall virulence factors are not attached to the pentaglycine crosslinks of staphylococcal peptidoglycan.

Once *srtA* was determined to be inactivated, the control and pYhcR plasmids where electroporated into the JH1787 strain. To determine if sortase A attached virulence factors are involved in YhcSR mediated survival of *S. aureus* the strains were assayed for survival in human blood. If sortase A attached virulence factor(s) are involved in YhcR mediated survival, inactivation of *srtA*

should eliminate the enhanced survival of the JH1787/YhcR-overproduction strain as over-produced cell surface virulence factors will no longer be translocated to and attached to the cell wall (283). As seen in Fig. 5-6, inactivation of *srtA* does not eliminate or impact the enhanced survival resulting from YhcR overproduction in strain JH1787 indicating sortase A attached cell surface virulence factors are likely not involved in YhcR mediated survival in human blood.

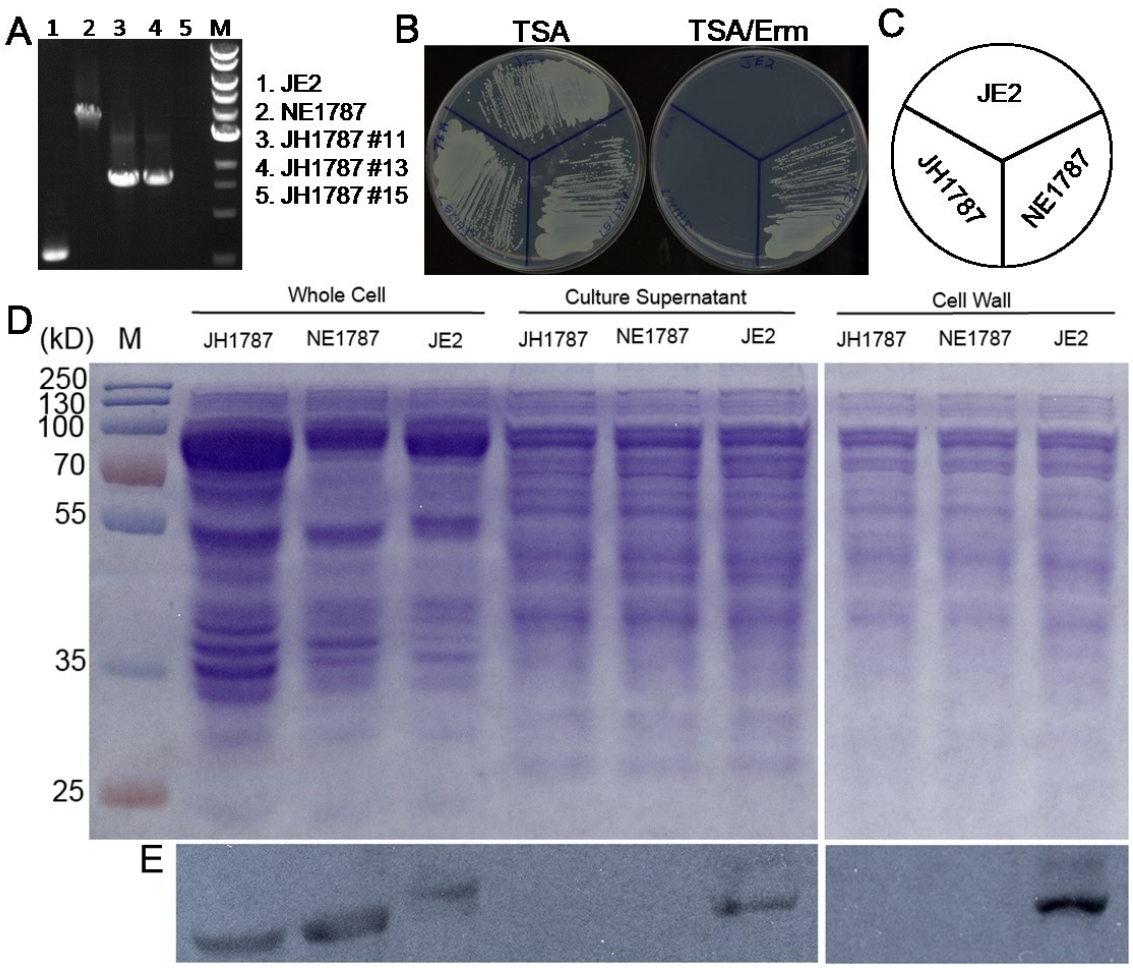

Figure 5-5 Deletion of *erm* gene in JH1787 and analysis of cellular protein A distribution. (A) Diagnostic PCR detection for loss of the *erm* gene from the transposon in *srtA*. **(B)** Streak plate of parental JE2 (erm^s), NE1787 (erm^r), and JH1787 (erm^s) on TSA and TSA/erythromycin showing JH1787 is now sensitive to erythromycin. **(C)** Diagram of strains streaked on TSA and TSA/Erm plates. **(D)** SDS-PAGE and **(E)** immunoblot of the cellular distribution of protein A in JE2, NE1787, and JH1787.

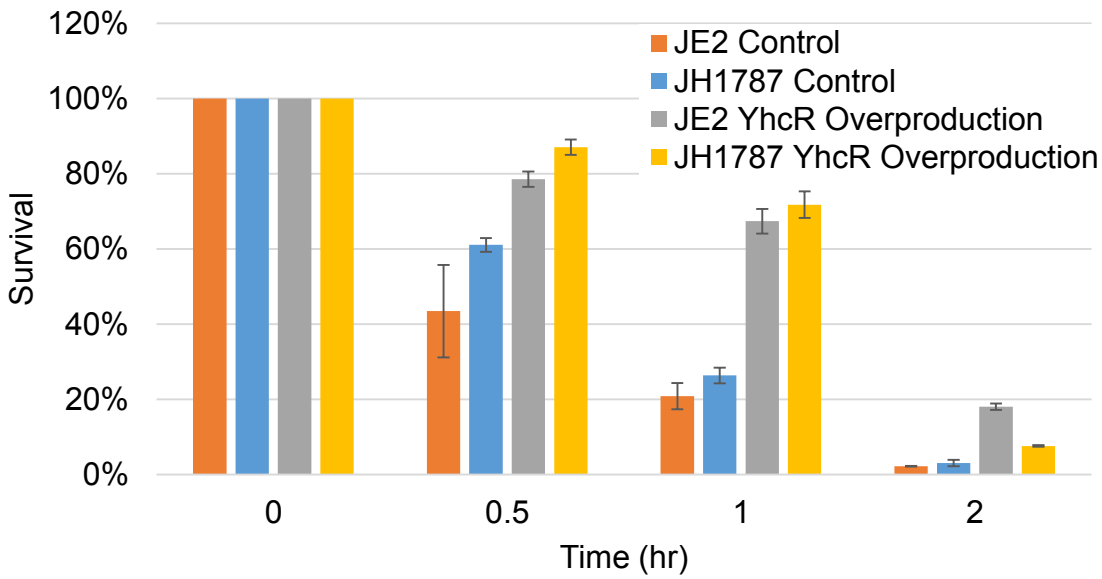

Figure 5-6 Inactivation of *srtA* does not prevent YhcR mediated enhanced survival. Percent survival of the *S. aureus* JE2 and JH1787 control and YhcR overproduction strains in human blood during ATc induction. The data represent the mean and standard deviation of two experiments. Strains were cultured overnight with the inducer ATc (250 ng/ml) and the following day diluted and inoculated into 500 ml of fresh blood with erythromycin, the inducer ATc (250 ng/ml) and incubated at 37°C.

5.4.3 Culture supernatants of YhcR overproducing *S. aureus* inhibit opsonophagocytic killing.

Long-term cell-mediated memory to *S. aureus* is minimal at best and the reasons for this are poorly understood (284). Thus, the humoral (antibody-mediated) and non-specific innate immunity (complement and phagocytic cells) are the primary and most important part of the immune system for controlling *S. aureus* infections (257–260). While the Gram-positive bacteria are believed to be relatively resistant to the complement-mediated membrane attack complex (285) because of their thick peptidoglycan cell-wall relative to Gram-negative bacteria, they are susceptible to opsonin- and antibody-mediated phagocytosis by neutrophils (258, 260).

In response to these phagocytic-mediating factors, *S. aureus* produces many factors that inhibit or degrade many of these components and aid in the dissemination of the bacteria (258, 284, 286). One mechanism of resistance is the proteolytic cleavage of complement and antibody factors, ultimately preventing phagocytosis (139, 286). To investigate if YhcSR regulated exported proteins contribute to the anti-phagocytic mechanisms of *S. aureus*; complement and serum were pre-incubated with concentrated culture supernatants of control and YhcR overproduction strains before being used in a modified opsonophagocytic killing assay.

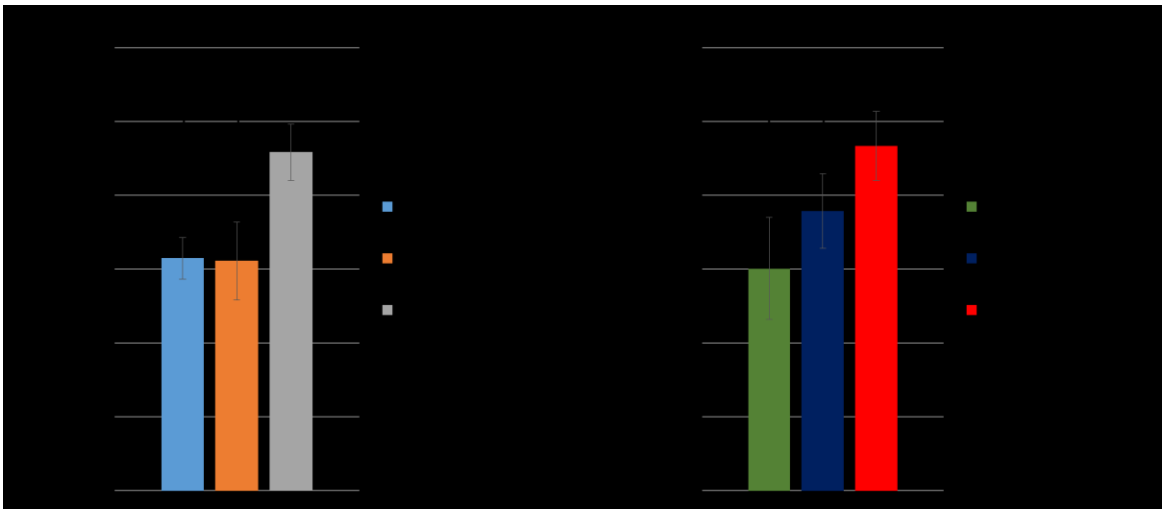

Figure 5-7 YhcSR regulated exported proteins inhibit complement and antibody mediated phagocytosis. Sterile TSB culture supernatants (Cult. Sup) of control and YhcR overproducing *S. aureus* (WYhcR) were concentrated 50-fold. The serum (A) and complement (B) components of the assay were pre-incubated 30 minutes with each culture supernatant and sterile concentrated TSB to allow *S. aureus* exported factors to act upon the immune system components before addition to the assay. Data presents the mean and standard deviation of 4 individual experiments.

Pre-incubating the serum fraction of the assay with the concentrated TSB vehicle control or culture supernatant of the control strain allowed for a similar percentage (~60%) of bacterial survival while pre-incubation of serum with the concentrated culture supernatant of the YhcR overproduction strain (WYhcR) resulted in a significant increase in the percentage of *S. aureus* that survived (~90%) in the assay compared to both the concentrated TSB and concentrated control culture supernatant (Fig. 5-7A). The data indicated that overproduction of YhcR resulted in inhibition of serum antibody-mediated phagocytosis. Pre-incubation of the complement fraction of the assay with the concentrated control culture supernatants produced an overall mean increase in the percentage of bacteria that survived compared to the concentrated TSB control (75% to 60%), but this increase was not significant. This is not unexpected, as *S. aureus* is

known to produce several complement inhibiting proteins (139, 260). The overproduction of YhcR resulted in a further, significant, increase of bacterial survival compared to concentrated TSB control (~90% to 60%) and concentrated control supernatant (~90% to 75%) (Fig. 5-7B). The data indicated that overproduction of YhcR contributed to complement inhibition and anti-phagocytic mechanisms of *S. aureus*. Taken together, the data indicate the YhcSR TCS is involved in regulation of exported proteins that inhibit opsonization and classical activation of the complement pathway.

5.4.4 Identification of over-produced exported proteins resulting from YhcR overexpression in *S. aureus.*

Inactivation of *srtA* revealed that it is unlikely that LPXTG cell-wall associated virulence factors are involved in the increased survival of the WYhcR strain. Furthermore, the opsonophagocytic assay revealed *S. aureus* exported proteins were involved in the YhcR mediated enhanced survival of *S. aureus.* As the second broad category of *S. aureus* virulence factors, exported proteins are secreted into the environment surrounding the bacterium. Many of these proteins are exported by the general secretion system (287, 288). Deletion of the main secretion protein, SecY, and some associated Sec proteins is not possible due to their essential nature in *S. aureus* (225, 281, 289–292), thus, examining how the Sec pathway contributes to YhcR mediated survival is problematic. To solve the issue of essentiality and directly identify which exported proteins were overproduced during YhcR overproduction, the exported proteins of the ATc induced control and WYhcR strains were precipitated from cell-free culture

supernatants. After denaturation and size separation by SDS-PAGE electrophoresis the exported proteins were visually compared (Fig. 5-8) and five proteins bands that were over-produced or bands that only appeared in the WYhcR lane were designated JH1-JH5 and processed for identification by mass spectrometry (See Material and Methods 5.3.5).

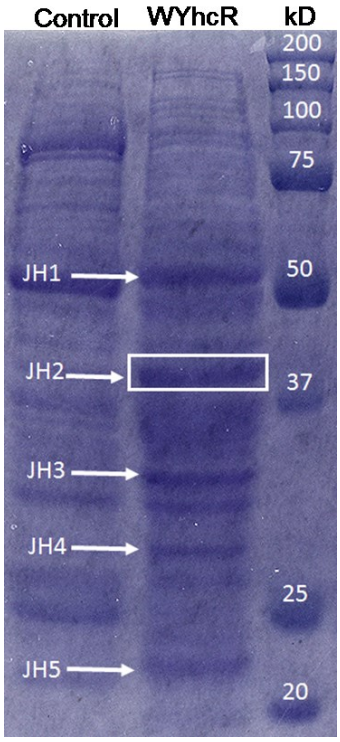

Figure 5-8 Comparative analysis of the exported protein profiles of the control and WYhcR strains. Each strain was induced with 250 ng/ml of inducer ATc overnight. The following day the bacterial cells were pelleted and the culture supernatant was filter sterilized. Proteins were precipitated with 100% EtOH overnight at 4°C. Precipitated proteins were separated by SDS-PAGE. Samples JH1-JH5 were chosen as the five most prominent bands relative the control.

Each processed sample had one protein that was the major component of the band (data not shown). The identified proteins were cross-referenced with published studies to identify proteins involved in innate immune suppression via inhibition of the humoral and/or innate cellular response. The parameters of the whole blood survival assay – human whole blood, defined volume, and a short assay period – suggested the YhcR-mediated enhanced survival mechanism likely inhibited the innate immune system response and not the adaptive immune system response. Published studies describing the immune system evasion properties of the predominant protein of sample JH2, the cysteine endopeptidase, staphopain B (SspB,) fell within this parameters. SspB is a known secreted protease of *S.*

aureus (293) and the *sspB* gene is co-transcribed in the same operon as *sspA* (V8 Protease) (294), a protease found to be up-regulated during growth in serum (295). More than 80% of the processed active form of SspB was identified by mass spectrometry.

To confirm the finding that SspB was up-regulated during YhcR overproduction, cell-free culture supernatants were prepared and the exported proteins were precipitated from the control and WYhcR strains without and with inducer ATc. As seen in Fig. 5-9A, the addition of ATc affects the protein profile of the control strain very little, while the addition of ATc to the WYhcR results in the appearance of a protein band that is similar in size to the processed active form of SspB, 27kD. Additionally, many protein bands are absent in the SDS-PAGE gel from the induced WYhcR strain which is consistent with previous reports that up-regulation of SspB (and SspA) resulted in degradation of other exported proteins (138, 293). As a positive control, the *sspB* gene was cloned into the same inducible expression vector (pYH4-*sspB,* WSspB). Very little extracellular protein is found in the supernatants of this strain as visualized by SDS-PAGE Coomassie Blue staining. Further confirmation of SspB up-regulation was carried out by immunoblot using chicken egg antibody specific for SspB (kindly provided by Alex Horswill (296)). In the control strain, SspB expression was low and appeared unaffected by the addition of ATc (Fig 5-9B). As increasing concentrations of ATc were added to to induce YhcR production, staphopain B was up-regulated in an ATc dependent manner in the WYhcR strain (Fig 5-9B). SspB was readily detectable in the induced WSspB strain as

well. The SspB specific antibody detected the various processed and degraded forms of the protein (297, 298). The data suggested a regulatory link between YhcSR and SspB production and indicated that the induced dose dependent increase in YhcR production resulted in a dose dependent increase in staphopain B production.

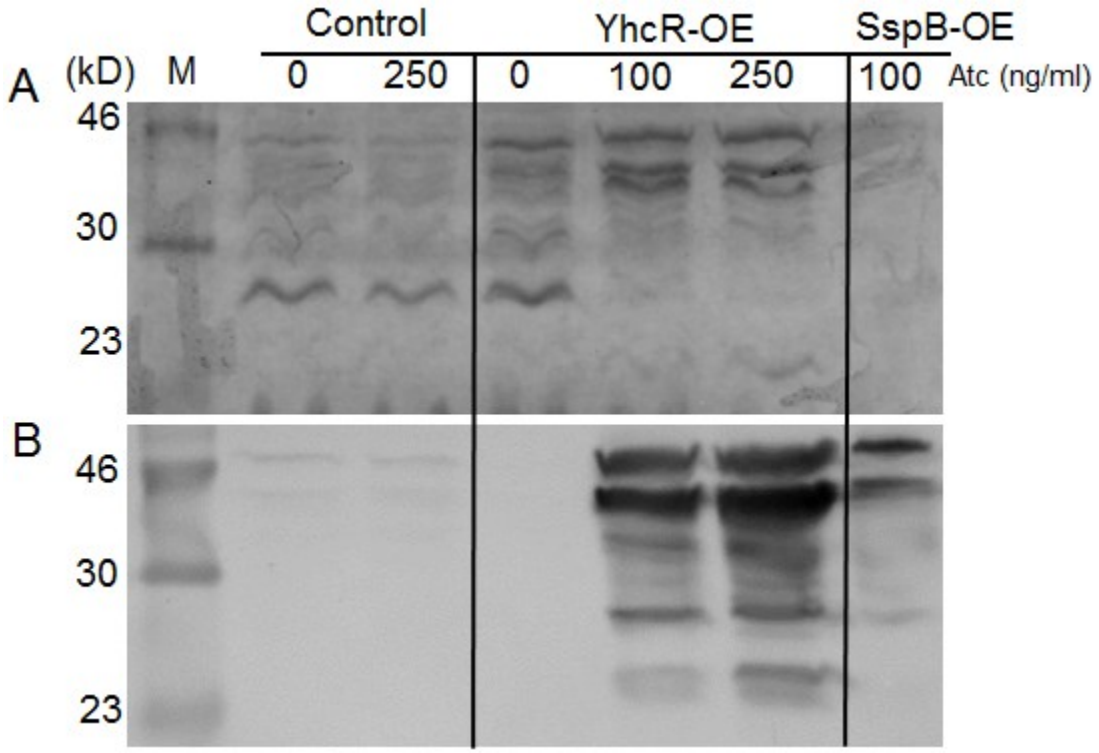

Figure 5-9 Induction of YhcR leads to up-regulation of SspB. (A) SDS-PAGE analysis of precipitated culture supernatant proteins from control and WYhcR without and with ATc and WSspB with ATc. **(B)** Western blot detection of SspB from precipitated culture supernatant proteins showing up-regulation of SspB with increasing induction of YhcR by inducer ATc.

5.4.5 Functional analysis of staphopain B expression.

To determine if the overproduced SspB was functional, the cell-free culture supernatants were assayed gelatin zymography, as SspB degrades collagen. Coomassie staining of the SDS-PAGE revealed the appearance of a single prominent band in the WYhcR lane and the disappearance of other proteins from supernatant (compare Fig 5-10A to B), similar to the finding in Fig. 5-9A. Gelatin zymogram analysis of the same samples revealed very little gelatin degradation in the control strain while a large, prominent band of gelatin degradation appeared in the WYhcR strain (Fig 5-10B). Importantly, the protein band that appeared in the WYhcR strain was at the same molecular weight as the band of gelatin degradation in the zymogram, suggesting the gelatin degradation is the result of this protein. The data highly suggested that the YhcR-mediated overproduction of the cysteine endopeptidase, SspB, is active and resulted in a large amount of protein degradation relative to the control strain culture supernatant. Taken together, the data indicated that overproduction of YhcR resulted in the functional overproduction of staphopain B.

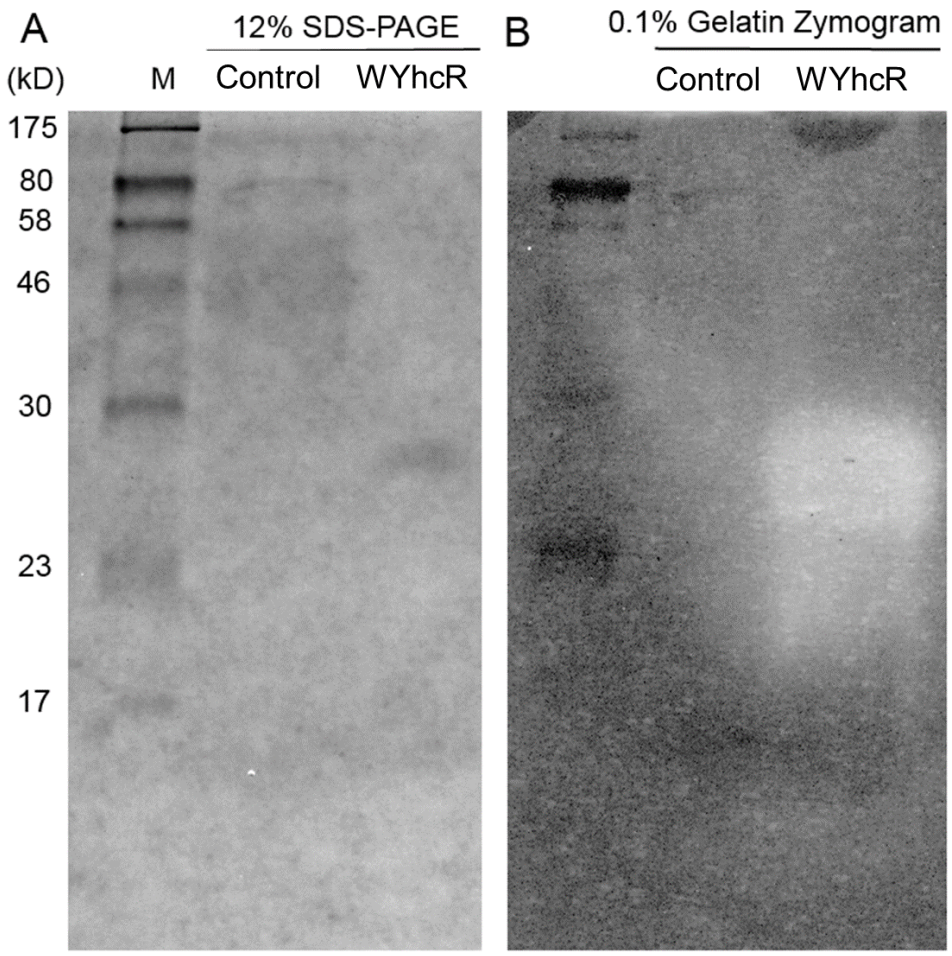

Figure 5-10 Coomassie stain and zymographic analysis of control and WYhcR. (A) Equal volumes of concentrated culture supernatant were resolved by 12% SDS-PAGE and proteins stained with Coomassie Blue. **(B)** Equal volumes of concentrated culture supernatant were resolved by 12% SDS-PAGE plus 0.1% gelatin and processed to remove SDS to allow for protein refolding. Following incubation the gel was stained with Coomassie Blue to reveal bands of gelatin degradation.

5.4.6 The *ssp* promoter is up-regulated during YhcR overproduction.

Staphopain B is produced from the middle gene of a three gene operon and is bordered downstream by *sspA,* encoding the V8 serine endopeptidase and upstream by *sspC* which encodes staphostatin B, a cytoplasmic inhibitor of SspB (294). To determine if the up-regulation of SspB occurs post-transcriptionally or if the entire *ssp* operon is up-regulated at the transcriptional level, the *ssp* promoter was cloned upstream of a promoterless-luciferase (*luxABCDE*) reporter cassette and electroporated into the control and WYhcR strain. As seen in Fig. 5-11, induction of YhcR expression with inducer ATc results in a 3-fold maximal increase in bioluminescence output from the P*ssp-lux* reporter relative to the control culture. Furthermore, P*ssp-lux* output was greater and sustained throughout the growth of WYhcR demonstrating that YhcR overproduction resulted in increased transcription from the *ssp* promoter.

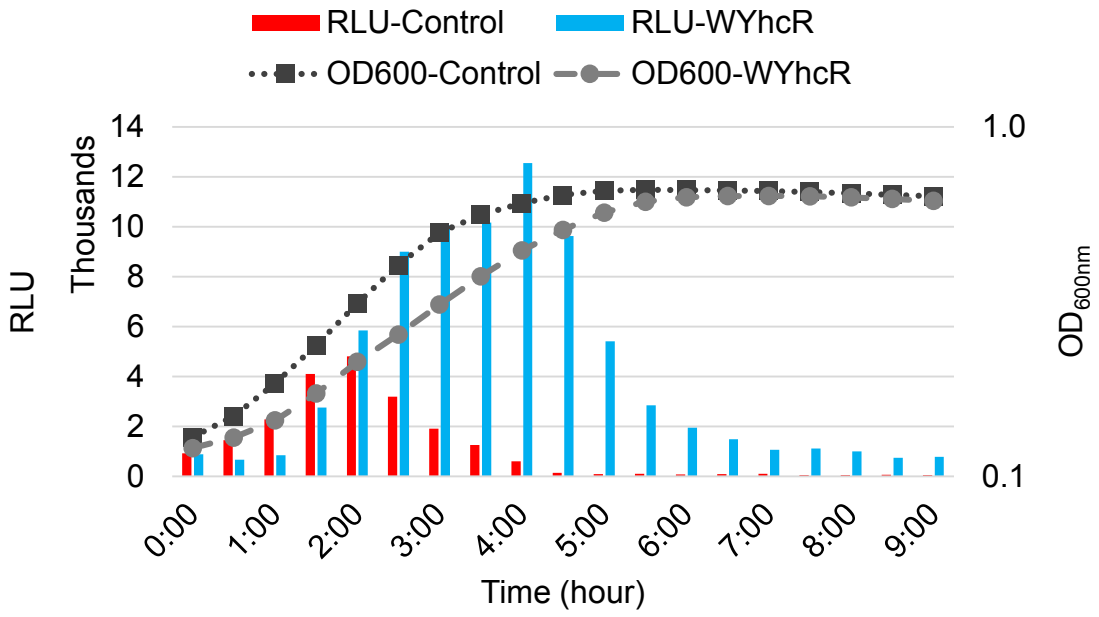

Figure 5-11 Promoter-reporter analysis of the *ssp* promoter during YhcR overproduction. The P*ssp-lux* reporter plasmid was electroporated into the control and WYhcR strains. Uninduced overnight cultures of each strain were diluted 1:300, incubated at 37°C with 25 ng/ml of inducer ATc. $OD600_{nm}$ and luminescence readings were measured every 30 minutes with 1 minute of mixing before each reading in a BioTek Synergy II spectrophotometer. The light intensity values for each time point are given as relative light units, Lum reading/$OD600_{nm}$ reading = RLU. The data presented are the mean of three independent experiments.

5.4.7 Up-regulation of the *ssp* promoter does not result from YhcR mediated regulation of *agr* and *sae*.

The *sspABC* promoter is regulated by several transcriptional regulators. Positive transcriptional regulation of the *ssp* promoter by the Agr TCS has previously been demonstrated (293, 298). Negative transcriptional regulation is mediated by virulence factor regulators SarA and the SaeRS two-component system as well as the alternative stress response sigma factor, σB (12, 293, 298, 299). A recent report on the YhcSR TCS provided evidence that YhcR binds the

promoter regions of *sae* and *agr* (129) and represses their expression in anaerobic culture conditions. Considering this information data experiments were developed to determine if overproduction of YhcR altered transcription from the *sae* and *agr* promoters in an aerobic luciferase-reporter plate assay. Induction of YhcR overproduction resulted in increased transcriptional reporter activity from the *agr* promoter (pCY1006) (Fig. 5-12A) and the *sae* promoter (pCY306) (Fig. 5-12B). Reporter activity for both promoters in the induced WYhcR strain relative to the control was increased from the mid-exponential phase onward. The data suggested YhcR positively regulates these two promoters in aerobic conditions.

To determine if up-regulation of the *sspABC* promoter was mediated by *sae* or *agr*, the control and pYhcR plasmids were electroporated into WCUH29*saeS* and WCUH29*agrA* mutants. These two strains were then electroporated with the P*ssp-lux* reporter plasmid. To confirm that regulation of the *sspABC* promoter in *S. aureus* WCUH29 was similar to previously published results for *S. aureus* SH1000 and UAMS-1 (12, 298), the basal reporter activity in the control strain for WCUH29 WT, WCUH29*saeS* and WCUH29*agr* in the absence of ATc was determined (Fig. 5-13A).

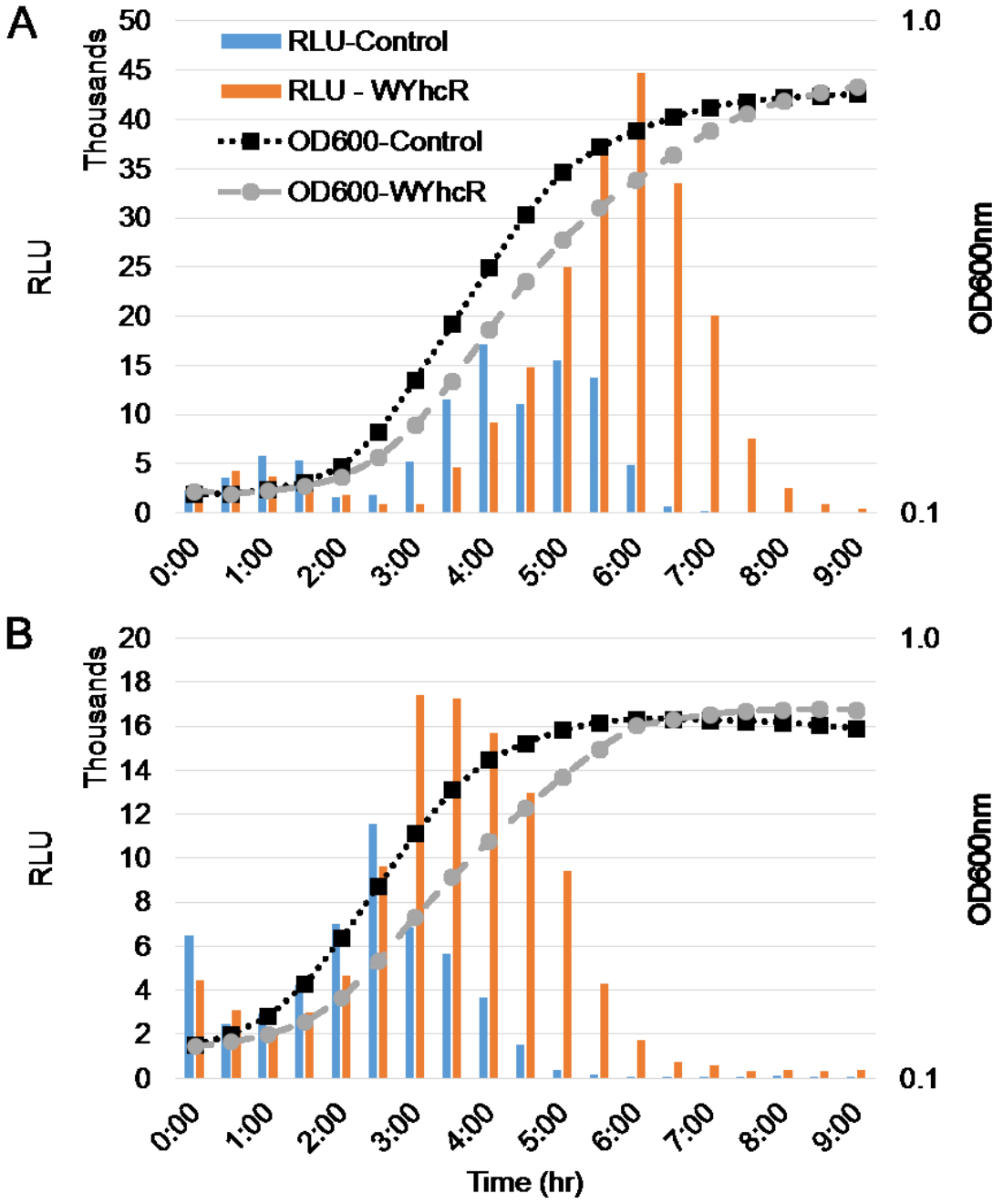

Figure 5-12 Promoter-reporter analysis of the *agr* and *sae* promoters during YhcR overproduction. (A) pCY1006 (P*agr-lux*) and **(B)** pCY306 (P*sae-lux*) were separately electroporated into the control and WYhcR strains. Uninduced overnight cultures of each strain were diluted 1:300, incubated at 37°C with 25 ng/ml of inducer ATc. $OD600_{nm}$ and luminescence readings were measured every 30 minutes with 1 minute of mixing before each reading in a BioTek Synergy II spectrophotometer. The light intensity values for each time point are given as relative light units, Lum reading/$OD600_{nm}$ reading = RLU. The data are the mean of three independent experiments.

Decreased and increased transcription of the *sspABC* promoter in the *agrA* mutant and *saeS* mutant, respectively, is consistent with published reports that Agr activated and Sae repressed transcription of the *sspABC* operon. The data suggested YhcSR are an activator of both *agr* and *sae* (Fig 5-12A and B), which are positive and negative regulators, respectively, of *sspABC*. If YhcR is solely responsible for increasing transcription of *sspABC* via indirect positive regulation of AgrA, deletion of *agrA* will eliminate any YhcR mediated increased transcription of *sspABC*. As indicated by the WCUH29*agr*/P*ssp-lux* reporter data in Fig. 5-13C, increased P*ssp* transcription continues to occur when YhcR is overproduced relative to the control, but maximal transcription of the *sspABC* promoter is slightly reduced in WCUH29*agrA*. The deletion of *agrA* does alter the growth phase expression pattern of the reporter, with maximal expression occurring earlier in the growth cycle of WCUH29*agrA*, suggesting that YhcR dependent up-regulation of the *agr* TCS may contribute to *sspABC* up-regulation in the late and post-exponential growth phases (compare 5-13B to C) (300). The incomplete loss of *sspABC* expression in WCUH29*agrA* suggested another transcription factor is involved or the *sspABC* up-regulation is mediated by direct YhcR positive regulation. As a known repressor of *sspABC*, deletion of *sae* should result in increased *sspABC* transcription and YhcR dependent up-regulation of *sae* should decrease expression of *sspABC*. To verify Sae is acting as a negative regulator, and not a positive regulator in WCUH29, the P*ssp-lux* plasmid reporter expression was monitored in the WCUH29*saeS* strain during YhcR overproduction.

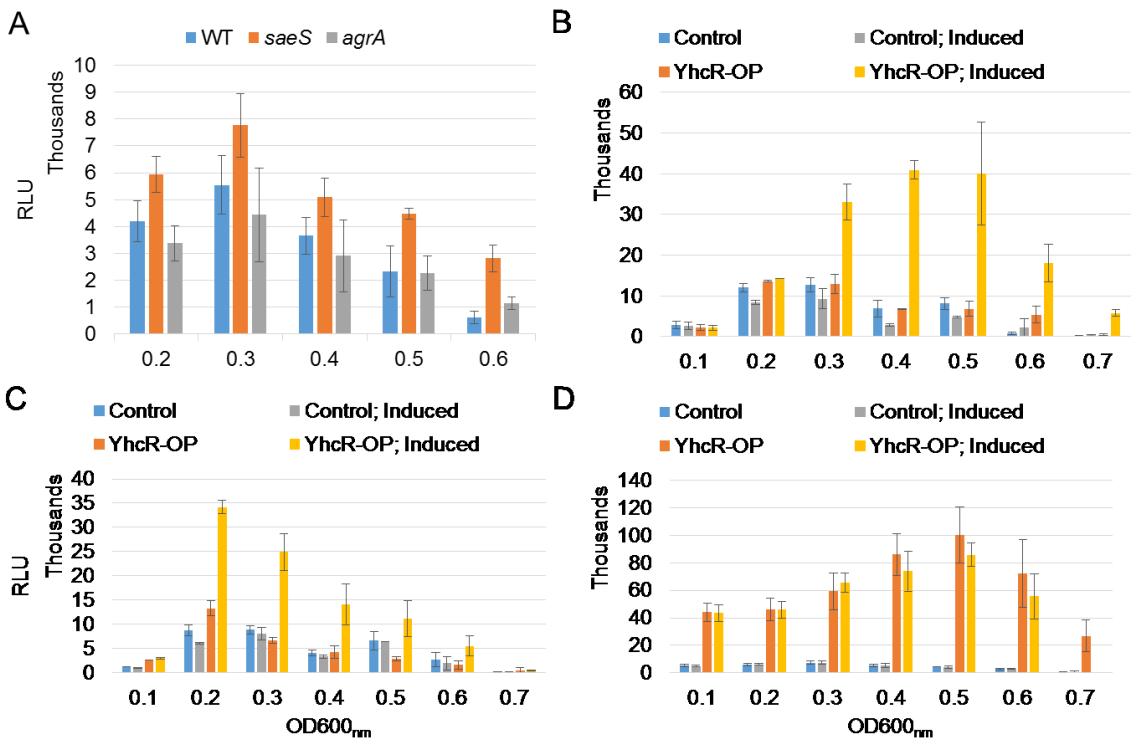

Figure 5-13 P*ssp-lux* plasmid reporter expression in WCUH29*agrA* and WCUH29*saeS* with and without YhcR overproduction. (A) Expression of P*ssp-lux* reporter in control WCUH29 WT, WCUH29*agrA* and WCUH29*saeS* strains in the absence of inducer ATc. P*ssp-lux* reporter expression without and with 25 ng/ml inducer ATc in control and YhcR overproduction (YhcR-OP) strains of **(B)** WCUH29 WT, **(C)** WCUH29*agrA*, **(D)** WCUH29*saeS*.

As seen in Fig. 5-13D, deletion of *saeS* resulted in a dramatic increase in P*ssp-lux* reporter expression, even without ATc induction of YhcR production, confirming that *sae* is indeed a repressor of *sspABC*. Furthermore, the high level of reporter expression in WCUH29*saeS* indicated that in the WCUH29 WT strain, P*ssp-lux* expression is intermediate, a likely result of direct YhcR positive regulation on the *sspABC* promoter and secondary YhcR regulation of *agr* and s*ae*, which in turn regulate P*ssp* positively and negatively, respectively, yielding an intermediate level of *sspABC* transcription overall in the wild-type *S. aureus* WCUH29.

5.4.8 YhcR binds the *sspABC* promoter.

The P*ssp*-reporter analysis indicated positive regulation of a*gr* by YhcR had a minor impact on the up-regulation of *sspABC*, but it could not account for all of the *sspABC* up-regulation that is produced during YhcR overproduction. Thus, it is likely that YhcR directly binds the *sspABC* promoter. To investigate this hypothesis an electrophoretic mobility shift assay (EMSA) was performed with purified YhcR-his and a P*ssp* probe. It was found that the mobility of the P*ssp* probe during native PAGE electrophoresis was increasingly retarded as the concentration of YhcR-his increased (Fig. 5-14, lanes 1-5), which indicated that YhcR bound the *sspABC* promoter probe. To determine the specificity of this binding, 100-fold excess of non-labeled specific P*ssp* probe was added to the reaction which caused incomplete labeled probe retardation, indicating that the non-labeled specific probe was competing with the labeled probe for the limited amount of YhcR-his (Fig. 5-14, compare lanes 4 and 6). To further confirm the specificity of the binding, a non-specific protein control was added (Fig 5-14, lane 7) and no apparent shift was seen, indicating the mere presence of protein did not inhibit the mobility of the P*ssp* probe. Lastly, the presence of 100-fold excess non-labeled, non-specific probe in the reaction of from two different internal DNA fragments of the *sspABC* operon did not interfere with the mobility of the YhcR-his/P*ssp* complex (Fig. 5-14, compare lanes 4 and 8-9). Taken together, the data indicated that YhcR specifically bound the upstream promoter region of the *sspABC* operon.

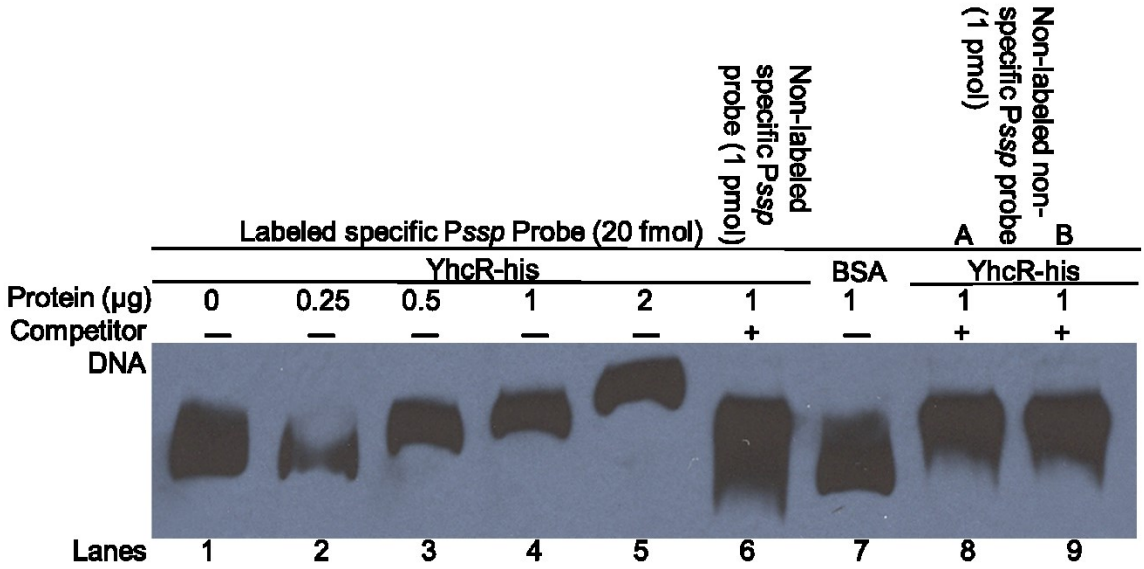

Figure 5-14 Electrophoretic mobility shift analysis of the *sspABC* promoter regulated by YhcR. The promoter region of the *sspABC* gene was obtained by PCR using 5' biotin-labeled primers. The mobility of the labeled promoter fragment without addition of YhcR is shown in the first lane. Different amounts of YhcR (0.25, 0.5, 1, 2 µg) were incubated with each labeled *sspABC* promoter probe in a 20 µl reaction volume. —, incubation with unlabeled specific competitor; +, incubation in the presence of 100-fold excess unlabeled specific competitor. BSA (1 µg) and a nonspecific internal gene probe (A) *sspA* and (B) *sspB* were used as nonspecific binding controls. Approximately 20 fmol of biotin-labeled *sspABC* promoter probe was used in each reaction mixture.

5.4.9 Deletion of *sspAB* partially eliminated YhcR-mediated enhanced survival in blood.

SspAB are involved in inhibiting many aspects of the innate immune response (139, 140, 262–264, 286). Furthermore, the data indicated that the YhcSR TCS regulated *sspABC* expression by direct positive transcriptional regulation. Lastly, overproduction of YhcR led to increased inhibition of opsonophagocytic killing by the exported proteins of *S. aureus,* of which SspAB are part of the exported *S. aureus* repertoire. Using an in-frame *sspAB* deletion plasmid (266), kindly provided by Alex Horswill, the *sspAB* genes were deleted from the *S. aureus* WCUH29 chromosome. The control plasmid, pYH4, and YhcR overproduction plasmid, pYhcR, were electroporated into *S. aureus* WCUH29Δ*sspAB* (JH114) creating JH214 (WCUH29Δ*sspAB*/pYH4) and JH314 (WCUH29Δ*sspAB*/pYhcR), respectively. Additionally, the *sspABC* operon was cloned in-frame downstream of the TetR inducible promoter in the pYH4 expression vector, creating pSspABC, and electroporated into JH114 to create JH414 (WCUH29Δ*sspAB*/pSspABC) for complementation and overproduction. The wild-type strains and *sspAB* mutant strains were assayed for their survival in human blood with ATc induction. As seen previously, overproduction of YhcR increased the percentage of CFU that survived throughout the 2 hour experiment (Fig 5-15, control vs. WYhcR). Deletion of *sspAB* had very little impact on the survival of *S. aureus* compared to the wild-type control (Fig 5-15, control vs. JH214). This was not unexpected as endogenous expression of individual proteases contribute only slight benefits to the overall survival of *S. aureus* and a significant decrease in fitness in blood and animal models is only seen when all

known proteases are deleted in *S. aureus* (296). That said, overproduction of YhcR in the *sspAB* deletion background did result in a significant decrease in the percentage of CFU that survived in the first half hour of the assay (Fig 5-15, WYhcR vs. JH314). After the first half hour, deletion of *sspAB* had minimal impact on the enhanced survival mediated by YhcR overproduction and by two hours, WYhcR and JH314 had similar percentage of surviving CFU. The data indicated that YhcR mediated overproduction of SspAB does contribute to the enhanced survival of YhcR over-producing *S. aureus* (WYhcR), but they contributed to survival early in the assay. The result further indicated other factors are involved in the enhanced survival of induced WYhcR over longer periods of incubation in human whole blood. The JH414 and WYhcR have similar percentage of CFU survival by hour 2 of the assay.

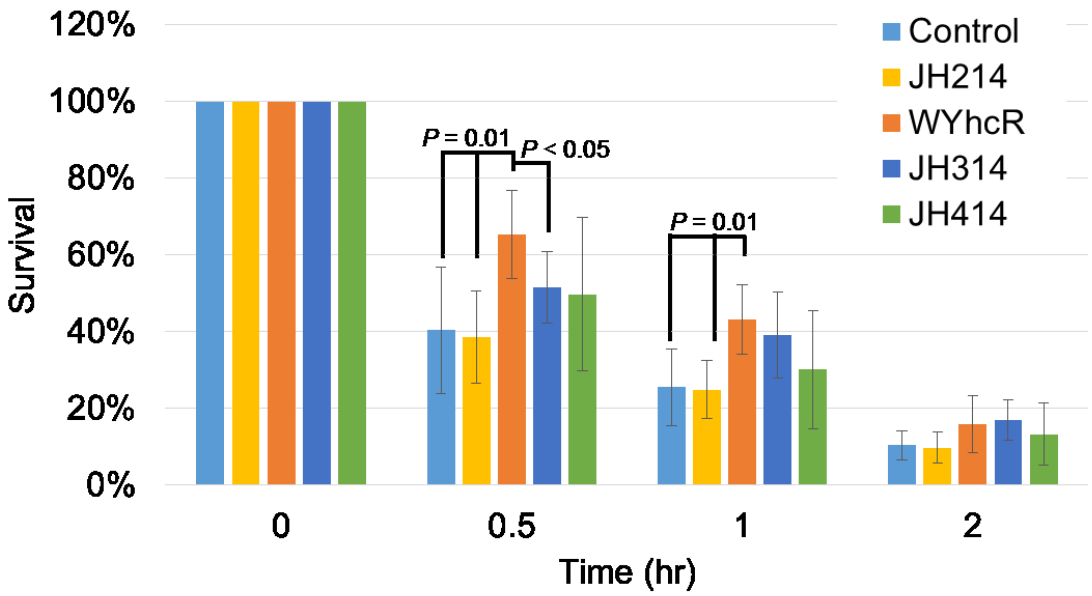

Figure 5-15 Deletion of *sspAB* partially eliminated YhcR-mediated enhanced survival in blood. Percent survival of the *S. aureus* WCUH29 wild-type and *sspAB* mutant strain with control and YhcR overproduction plasmids in human blood during ATc induction. The data is the mean and standard deviation of four experiments. Statistical analysis conducted with Student's T-Test; P ≤ 0.05. Cultures of *S. aureus* strains were cultured overnight with the inducer ATc (250 ng/ml) and the following day diluted and inoculated into 500 ml of fresh blood with erythromycin and the inducer ATc (250 ng/ml) and incubated at 37°C.

5.5 Discussion

To investigate and determine if a relationship between pathogenesis and the YhcSR TCS existed, the inducible *yhcS* antisense RNA and YhcR overproduction mutants were analyzed for how well they survived in human whole blood. It was not unexpected to find depletion of YhcSR inhibited survival in human blood. The decreased survival is likely related to inhibited cell growth from YhcSR depletion, as shown to occur *in vitro* (128). Conversely, overproduction of YhcR enhanced the survival of *S. aureus*. This was unlikely to be related to growth rate, as we know that YhcR overproduction does not increase the growth rate of *S. aureus,* at least in TSB cultures.

Staphylococcus aureus produces numerous LPXTG cell-surface associated and exported virulence factors (8, 301). To determine which group of factors was involved in the enhanced survival mediated by YhcR overproduction a *srtA* mutant/YhcR-overproduction strain was analyzed and it was found to survive well as the wild-type JE2/YhcR-overproduction strain in human blood which suggested *srtA* attached virulence factors were not involved in the enhanced survival phenotype. To assess the contribution of exported proteins to the enhanced survival phenotype, a modified HL-60 opsonophagocytic killing assay was developed. Pre-incubation of culture supernatant from the induced WYhcR strain with complement components and human serum significantly enhanced survival of *S. aureus* in the assay relative to the TSB control alone and control strain culture supernatant, which suggested YhcR-regulated exported proteins were inhibiting opsonin-based phagocytosis of *S. aureus* in blood.

Comparative analysis of the exported protein profile of the induced control and WYhcR strains revealed several protein bands to be highly overproduced in the WYhcR culture supernatant. Using mass spectrometry for peptide analysis and immunoblotting, staphopain B, was identified as one of the highly enriched proteins in the WYhcR culture supernatant. The operon of *sspABC* encodes the V8 protease and staphopain B, both of which are known to promote survival and inhibit opsonophagocytosis (139, 140, 263, 264). This information corresponded well with the finding that WYhcR culture supernatants inhibited opsonophagocytic killing of *S. aureus* relative to the control supernatants.

Using a P*ssp-luxABCDE* reporter plasmid it was shown that transcription from the *sspABC* promoter was up-regulated relative to the control by overproduction of YhcR which led to overproduction of SspB, as detected by immunoblotting. Furthermore, the transcriptional up-regulation is a direct result of YhcR binding to the P*ssp*, as confirmed by gel-shift analysis, and not through *sae* or *agr*, other known direct regulators of P*ssp.*

Overproduction of YhcR in the absence of *sspAB* (WCUH29Δ*sspAB*) only significantly decreased survival of *S. aureus* in the first 30 minutes of incubation in human blood, which was unexpected as YhcR overproduction promoted survival throughout the 2 to 3 hour experiment time-frame. An explanation for this observation lies in the fact that proteases, including SspA and SspB, modulate the stability and abundance of staphylococcal extracellular and cell-associated virulence factors through proteolytic cleavage (138, 296). Therefore, it is possible other virulence factors involved in opsonophagocytic inhibition, protein A for

example, may have accumulated in the absence of the proteases during the assay period in the defined volume of blood and possibly complemented the loss of the SspAB proteases.

The YhcSR TCS system regulates gene expression in response to the presence or absence of oxygen, and possible reactive oxygen species (129). The regulation of *sspABC* by YhcSR if of interest in the context of biofilm formation and stability and abscess formation, in addition to its apparent role in survival in blood. Biofilms and wound sites are known to have varying degrees of hypoxia (302–304) thus, it is conceivable that YhcSR may regulate expression of SspAB in response to the oxygen pressure levels in the microenvironment. This regulation likely has implications in biofilm formation and stability, extracellular matrix destruction and wound healing, as well as neutrophil infiltration and response to infections (139, 261–263, 266, 296, 305, 306). Further investigation is needed to define the role of YhcSR in relation to SspAB production in these microenvironments and how YhcSR regulation of *sspABC* impacts the pathogenesis of *S. aureus* in these environments.

The data presented in this chapter indicate the YhcSR TCS of *S. aureus* positively regulates the expression of *sppABC* at the transcriptional level. Using an YhcR overproduction mutant, it was identified that the *sspABC* promoter is up-regulated and at least one protease of the operon, SspB, is overproduced. The protease overproduction that resulted from YhcR overproduction partially enhanced survival of *S. aureus* in human whole blood; linking the YhcSR TCS to anti-opsonophagocytic killing mechanisms of *S. aureus*.

Chapter 6 : The YhcSR two-component system likely positively regulates the staphyloxanthin biosynthetic operon, *crtOPQMN*

6.1 Overview

The golden pigment of *Staphylococcus aureus* distinguishes the species from other staphylococcal species and is the product of a series of biosynthetic steps that produce a unique C(30) golden carotenoid, staphyloxanthin. Staphyloxanthin acts as an antioxidant and impairs reactive oxygen species mediated killing within the phagosome of professional phagocytes and thus, promotes virulence and survival of *S. aureus*.

It was observed that the golden pigment of a strain of *S. aureus* ectopically expressing YhcR from a chromosomally integrated *spac*-promoter cassette was noticeably increased compared to the wild-type strain. Subsequent analysis revealed that depletion of YhcSR by *yhcS* antisense RNA and overproduction of YhcR decreased and increased by 50%, respectively, the amount of staphyloxanthin produced by *S. aureus.* Staphyloxanthin is important for hydrogen peroxide resistance of *S. aureus*. Depletion of YhcSR and overproduction of YhcR decreased and increased, respectively, the hydrogen peroxide susceptibility of *S. aureus* significantly. Furthermore, using a whole blood survival assay, the genetic elimination of staphyloxanthin during YhcR overproduction abolished the protective phenotype of increased staphyloxanthin production, which is consistent with staphyloxanthin's role in impairing reactive oxygen species mediated killing of *S. aureus* by neutrophils.

6.2 Introduction

The distinctive golden pigment of *Staphylococus aureus* is the biosynthetic product of *crtOPQMN* and *aldH* (307, 308). This six enzyme biosynthetic pathway's final product is the golden C(30) triterpenoid carotenoid, α-D-glucopyranosyl 1-*O*-(4,4'-diaponeurosporen-4-oate) 6-*O*-(12-methyltetradecanoate), or more simply, staphyloxanthin (STX) (309). The antioxidant properties of STX were first reported by Clauditz et al. (310) and several follow-up studies have confirmed this initial report and identified STX as an important virulence factor for intracellular phagocyte survival of *S. aureus* (311) and is linked to resistance to reactive oxygen species (ROS) produced by the NADPH oxidase system within the phagocyte phagosome (312).

Currently, it is known that the stress response alternative sigma factor B regulates STX production, as a σ^B deletion mutant is white and lacks STX. This regulation is likely direct as a consensus σ^B DNA binding motif was identified in the *crt* promoter, but direct interaction has not been confirmed (313). Additionally, the cold shock protein A, CspA, is a positive regulator, likely in a σ^B dependent manner (314). The metabolism of *S. aureus* is also known to influence pigment production, as mutants with a defective tricarboxylic acid cycle or inability to perform oxidative phosphorylation showed increased pigmentation and the response was dependent on expression of σ^B (315).

In this chapter data are presented that show the YhcSR TCS directly transcriptionally activates the *crtOPQMN* operon and modulates STX carotenoid

production. Moreover, YhcSR's regulation of staphyloxanthin influences *S. aureus* susceptibility to hydrogen peroxide and survival in human whole blood.

6.3 Materials and methods

6.3.1 Bacterial strains, plasmids and growth media.

The bacterial strains and plasmids used in this study are listed in Table 6-1. The *S. aureus* cells were cultured in trypticase soy broth (TSB) at 37˚C with shaking. Transformants containing recombinant plasmids were selected on trypticase soy agar (TSA) containing chloramphenicol (10 µg/ml) or erythromycin (5 µg/ml) for *S. aureus*. Sheep's blood agar plates were obtained from BD. For anaerobic growth, TSA plates were placed in an oxygen-free, nitrogen-hydrogen gas mixture COY chamber and incubated room temperature.

6.3.2 Construction of *S. aureus* WCUH29 P*spac-yhcS* and –*yhcR* strains.

S. aureus WCUH29 P*spac-yhcS* and –*yhcR* were constructed by phage transducing the pFF71-*yhcS* or –*yhcR* chromosomal fragment from SASJ104 and SASJ204, respectively (128, 316). Briefly, SASJ104 and SASJ204 were cultured and used to make separate φ11 lysates. The lysates were then used to infect wild-type *S. aureus* WCUH29 and transductants were selected on TSA with chloramphenicol. The pFF71 plasmid site-specifically integrates into the *S. aureus* chromosome at the φL54α *attB* site located in the lipase (*geh*) gene (317). Transductants that were Cm^r and lipase negative on Spirit Blue Agar plates with Lipase Reagent (BD) were cultured and genomic DNA was extracted and screened by diagnostic PCR using the PspacFor/YhcROE-Rev primer set,

listed in Table 6-2, to specifically identify colonies that had recombined the pFF71-*yhcS* or –*yhcR* chromosomal fragment into the genome.

6.3.3 Extraction of staphyloxanthin from *S. aureus* strains.

The *S. aureus* strains were cultured in 5 ml of TSB in a 50 ml conical vial at 37°C and shaking at 220 RPM with respective inducers; 200 µM IPTG or 500 ng/ml ATc. After 24 hours the $OD600_{nm}$ of each culture was read in 200 µl duplicates using a BioTek Synergy II spectrophotometer. One ml of culture was carefully washed twice in PBS. The pelleted cells were resuspended in one ml of 100% methanol with pipetting and vortexing until all large clumps of cells were dispersed. The extractions were placed in a rotisserie incubator and rotated end-over-end at room temperature for 1 hour. Cellular debris was pelleted and the $OD450_{nm}$ for each methanol extraction was measured using 200 µl aliquots in duplicate on the BioTek spectrophotometer. The fold change was calculated as Normalized Mutant/Normalized control. To normalize STX concentration, the $OD450_{nm}$ reading was divided by the respective $OD600_{nm}$ reading for each sample (normalized STX concentration = $OD450_{nm}/OD600_{nm}$). The experiment was repeated at least three times.

6.3.4 Hydrogen peroxide susceptibility assay.

Hydrogen peroxide (H_2O_2) was added to 1.5% final concentration in PBS and $2x10^8$ CFU were incubated at 37°C for 1 hour. Serial dilutions were plated on trypticase soy agar (TSA) for enumeration of surviving CFU. Percent survival was calculated as surviving #CFU/ input #CFU multiplied by 100, $(\#CFU_f/\#CFU_i)*100$.

6.3.5 Electrophoretic mobility shift assay.

The *crt* promoter primers are listed in Table 5-2. The promoter fragment was obtained by high-fidelity PCR. The PCR was loaded into a 2% agarose gel. The sample was electrophoresed, and the gel was stained with ethidium bromide. The promoter fragment was removed from the gel and purified using a NucleoSpin Gel Clean-up kit (Macherey-Nagel).

For the electrophoretic mobility shift assay (EMSA) a 5% native TBE PAGE gel was prepared and pre-run using 0.5X TBE. Samples were prepared in 1X PBS, with 50 ng of total *crt* or *ssp* promoter probe, 50 ng/µl poly (dI•dC), and 2.5% glycerol. 1 µg of YhcR-His, specific binding protein, and 10 µg of BSA, non-specific binding protein, was added where indicated .Ultrapure water was added so that all reaction volumes totaled 20 µl. The reactions were incubated at room temperature for 20 minutes and then 5 µl of 5X loading buffer was added to each reaction. 20 µl of each reaction was loaded in a pre-run 5% TBE native polyacrylamide gel and electrophoresed at 100V for two hours at 4°C. After electrophoresis, the gel was incubated in DI water containing 5 µg/ml ethidium bromide for 10 minutes. The gel was washed three times with DI water and the mobility of DNA probes was visualized with a long wave UV light and photographed.

Table 6-1 Bacterial strains and plasmids

Strain	Description	Source
SASJ104	RN4220 *geh::*pFF71-*yhcS*; Cm^r	(128)
SASJ204	RN4220 *geh::*pFF71-*yhcR*; Cm^r	(128)
WJH104	WCUH29 *geh::*pFF71-*yhcS*; Cm^r	This Study
WJH204	WCUH29 *geh::*pFF71-*yhcR*; Cm^r	This Study
WCUH29/pYH3	WCUH29 antisense control strain with empty pYH3; Erm^r	(128)
JSAS909	WCUH29 with pYJY909; Erm^r	(128)
WCUH29/pYH4	WCUH29 protein overproduction control with empty pYH4; Erm^r	This Study
WYhcR	WCUH29 with pYH4-*yhcR*; Erm^r	This Study
JH113	WCUH29 with in-frame deletion of *crtM*	This Study
JH213	WCUH29Δ*crtM* with pYH4; Erm^r	This Study
JH313	WCUH29Δ*crtM* with pYhcR; Erm^r	This Study

Plasmids	Description	Source
pYH3	Shuttle vector with a TetR regulated inducible promoter; Erm^r	(128)
pSAS909	pYH3 with *yhcS* antisense downstream of TetR promoter; Amp^r, Erm^r	(128)
pYH4	pYH3 with Amp^r removed; Erm^r	(270)
pYhcR	*yhcR* cloned downstream of pYH4 TetR promoter; Erm^r	This Study
pKOR1	Temperature sensitive inducible allelic exchange plasmid for *S. aureus*; Cm^r	(191)
pKOR1-*crtM*	pKOR1 with in-frame *crtM* upstream/downstream deletion region; Cm^r	This Study

Table 6-2 Oligonucleotide sequences

Primers	Sequence
Pspacfor	5'-GCTTGAATTCATTCAGAACGCTCGGTTGCC-3'
YhcROE-rev	5'-TTGGCGCGCCCTATTTTATAGGAATTGTGAATTG-3'
crtM-pKOR1-L-For	5'-GGGG ACAAGTTTGTACAAAAAAGCAGGCT CAGCAAGTTCCAGTAGATGTCATTG-3'
crtM-pKOR1-L-Rev	5'-CATACTAGTCCTCCTATATTGAAATGCC-3'
crtM-pKOR1-R-For	5’-Phos- CATAGAATATAGGTGGTTGAATAATGAAGATTG-3'
crtM-pKOR1-R-Rev	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTG ATCTGTCACATCAATATCTATACCG-3'
Pcrt For	5’-CTAATGGTTATGCATCAGGAAGTAAC-3’
Pcrt Rev	5’-CTAAATTGAATCACTCTCAATCATACTGAC-3’

6.4 Results

6.4.1 Ectopic y*hcR* expression made *S. aureus* colonies more golden in color.

During the construction of what were intended to be IPTG inducible P*spac* regulated *yhcS* (WJH104) and *yhcR* (WJH204) *S. aureus* WCUH29 strains (128, 200) it was observed that the golden color of the ectopically expressing *yhcR* colony was remarkably enhanced relative to the wild-type *S. aureus* WCUH29 strain and ectopically expressing *yhcS* strain. This phenotype was seen on TSA plates (Fig. 6-1A) and sheep's blood agar plates (Fig. 6-1B). The enhanced golden pigment of the WJH204 strain was observed when the strain was grown aerobically; when grown anaerobically, the golden pigment was absent in all strains (Fig. 6-1C).

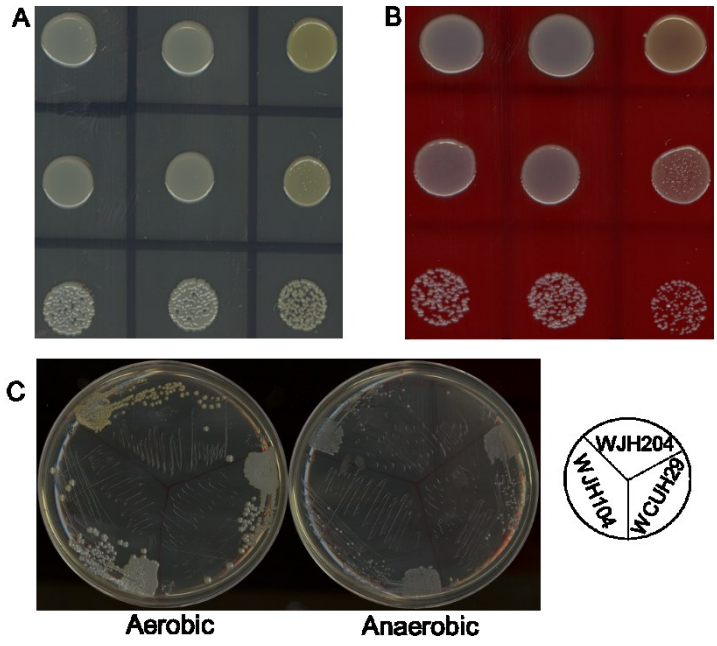

Figure 6-1 Ectopic expression of YhcR enhances the golden color of *S. aureus*. Overnight cultures of the strains were serially diluted and 10 µl of the 10^{-3}, 10^{-5}, and 10^{-7} dilutions were plated on **(A)** TSA and **(B)** 5% sheep's blood agar plates and incubated 24 hours at 37°C. **(C)** Each strain was streaked on TSA plates and incubated aerobically or anaerobically. The diagram on the right outlines the placement of the strains on the plates.

6.4.2 Altering the expression levels of YhcR altered the amount of golden pigment, staphyloxanthin, produced by *S. aureus* WCUH29.

Synthesis of the golden pigment of *S. aureus* is encoded by the *crtOPQMN* operon plus the *aldH* gene (308). These six enzymes synthesize the golden carotenoid, staphyloxanthin (STX), of *S. aureus* in a multistep biosynthetic pathway (307, 309, 318). The STX pigment has shown to be important for cellular protection from UV radiation and oxidants (310) and to be a virulence factor, protecting the bacterium from oxidants during phagocytosis (312)

To confirm that alteration of the level of YhcSR production modifies the amount of STX produced in *S. aureus* cells, the carotenoid was extracted using methanol after 24 hours of aerobic growth and STX production was compared from YhcSR deplete cells and cells ectopically producing YhcR relative to the respective control cells. Depletion of YhcSR by induction of *yhcS* antisense RNA (128) resulted in a 50% decrease in the amount of STX compared to control cells (Fig. 6-2A). Conversely, ectopic expression of YhcR (WJH204) produces a 50% increase in the amount of STX produced by the bacteria compared to the control cells (Fig. 6-2B). The data indicated that altering the level of YhcSR produced altered the amount of STX produced in *S. aureus* cells.

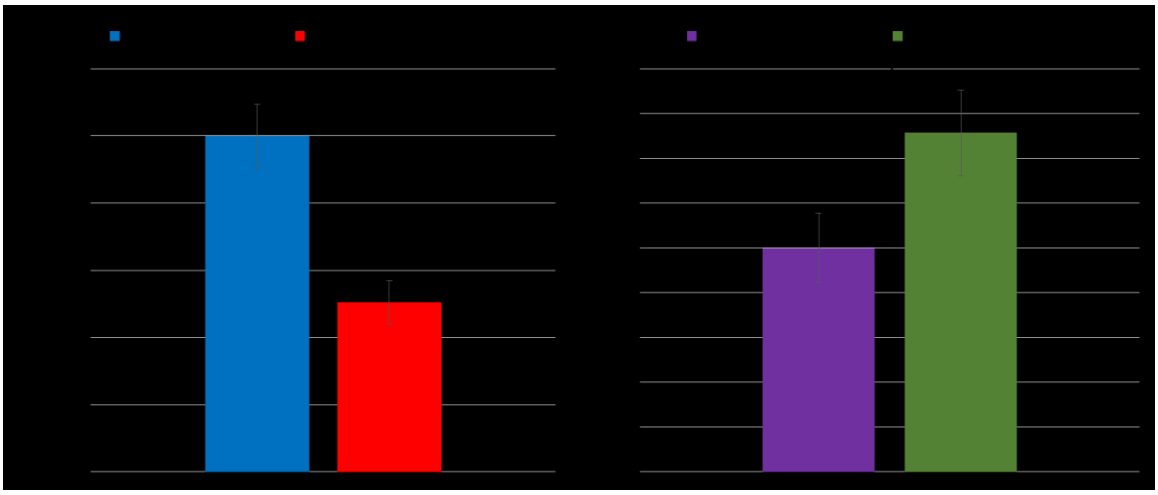

Figure 6-2 Altering the expression level of YhcR altered the amount of staphyloxanthin produced by *S. aureus*. (A) WCUH29/pYH3 and **(B)** WCUH29/*yhcS* antisense RNA (JSAS909) **(B)** WCUH29 and WCUH29::P*spac-yhcR* (WJH204); induced with 500 ng/ml ATc and 200 µM IPTG, respectively. The data represent the mean ± standard deviation of at least three experiments.

6.4.3 Altering the expression levels of YhcR altered susceptibility of *S. aureus* WCUH29 to killing by hydrogen peroxide.

The membrane bound staphyloxanthin carotenoid protects *S. aureus* from ROS, such as hydrogen peroxide (310, 312). To determine if altering the production of YhcR alters susceptibility of *S. aureus* to hydrogen peroxide killing, the strains were assay for survival in a H_2O_2 susceptibility assay. It was found that depleting *S. aureus* of YhcSR by *yhcS* antisense RNA significantly reduced survival after one hour of incubation in 1.5% H_2O_2 (Fig.6-3A). Conversely, overproduction of YhcR resulted in significant protection from killing by H_2O_2 (Fig 6-3B). The data indicated that YhcSR contributed to protection from ROS, likely through regulation of STX biosynthesis.

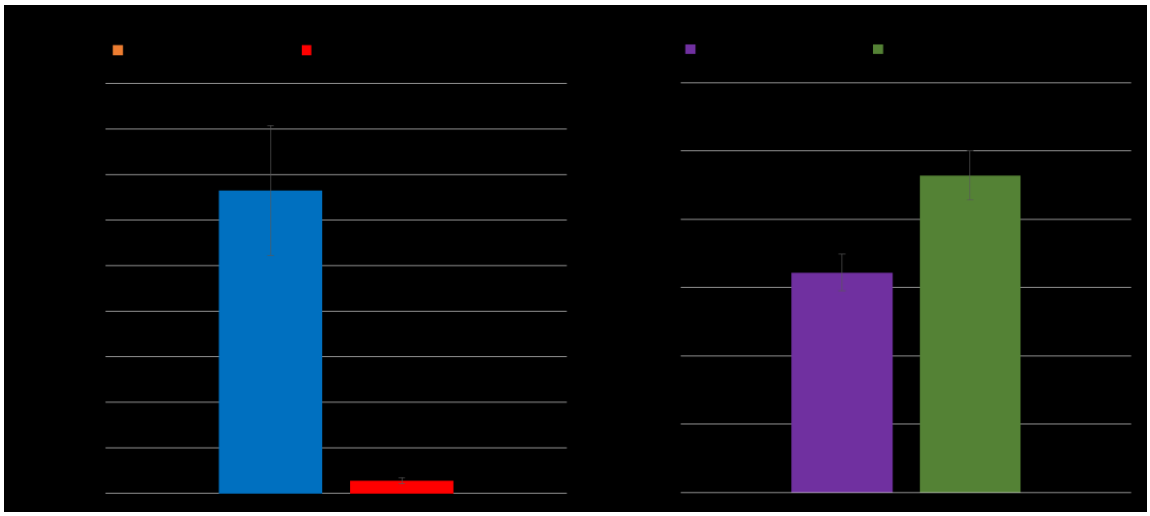

Figure 6-3 Altering the expression of YhcR alters *S. aureus* susceptibility to H_2O_2. (A) control and *yhcSR* antisense RNA strains; induced with 500 ng/ml ATc. **(B)** control and YhcR overproduction strain (WYhcR); induced with 250 ng/ml ATc. Approximately $5x10^8$ CFU were incubated in PBS with 1.5% hydrogen peroxide at 37°C for 1 hour. The percent survival was calculated as $(\#CFU_f/\#CFU_i)*100$. Statistical analysis conducted with Student's T.Test. The data represents the mean ± standard deviation of three experiments.

6.4.4 YhcR likely directly binds the upstream promoter region of *crtOPQMN.*

To determine if the increased production of STX was due to direct positive regulation by YhcR, an electrophoretic mobility shift assay (EMSA) with ethidium bromide staining was performed to determine if YhcR was capable of binding the upstream promoter region of *crtOPQMN*. As seen in Fig. 6-4, the addition of YhcR-His to the reaction resulted in electrophoretic mobility retardation of the P*crt* probe. The addition of a non-specific protein, BSA, caused no such retardation. The previously determined YhcR regulated promoter of *sspABC* was used as a positive control and shows a similar retardation pattern. The data suggested that YhcR binds the *crt* promoter and that the YhcSR TCS is a positive regulator of the *crtOPQMN* operon.

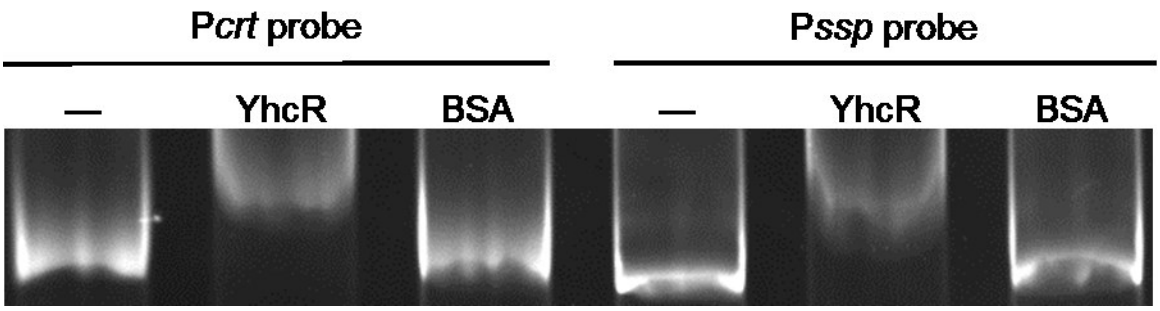

Figure 6-4 Electrophoretic mobility shift analysis of the *crtPQOMN* promoter regulated by YhcR. 50 ng of each promoter probe was added to reactions. Either no protein was added (—), 1 µg of YhcR-His, or 10 µg of BSA was added to each reaction and incubated for 20 minutes at room temperature. Reactions were then electrophoresed on a 5% native 0.5X TBE PAGE gel for 2 hours at 100V followed by staining with ethidium bromide to visualize the DNA.

6.4.5 Elimination of staphyloxanthin in *S. aureus* during YhcR overproduction eliminateed YhcR-mediated enhanced survival in human blood.

The presence of STX has been shown to decrease the killing of *S. aureus* by purified neutrophils and in whole blood. The decreased susceptibility was linked to protection against ROS produced by the phagocyte NADPH oxidase system (312, 319). Circulating neutrophils are a major component of blood phagocytes. Previously, it was identified that YhcR overproduction significantly enhanced the percentage of *S. aureus* that survived in human whole blood. To determine if the YhcR mediated overproduction of STX was, at least partially, responsible for the increased survival of the WYhcR strain the first gene in the STX biosynthetic operon, *crtM*, was deleted. The deletion created an *S. aureus* strain that lacked capability of producing any carotenoid intermediates or STX (Fig. 6-5A, JH113). The pYH4 and pYhcR plasmids were subsequently electroporated into the JH113 creating JH213 and JH313, respectively.

The deletion of *crtM*, resulting in STX-less *S. aureus* resulted in a slight decrease, on average, in survival for the mutant throughout the experiment, but was only significant at the one hour time point of incubation (Fig 6-5B, control vs. JH213). As seen previously, induction of YhcR overproduction resulted in significantly enhanced survival of *S. aureus* during incubation in human blood (Fig 6-5B, WYhcR). The enhanced survival that resulted from YhcR overproduction was completely eliminated in the *crtM* mutant (Fig 6-5B, JH213 vs. JH313). The data indicated that YhcR mediated overproduction of STX significantly contributed to the enhanced survival of *S. aureus* during YhcR overproduction. The data also suggested that the enhanced survival of the WYhcR strain is the result of improved phagocyte oxidative burst resistance due to overproduction of STX as a direct transcriptional result of YhcR overproduction.

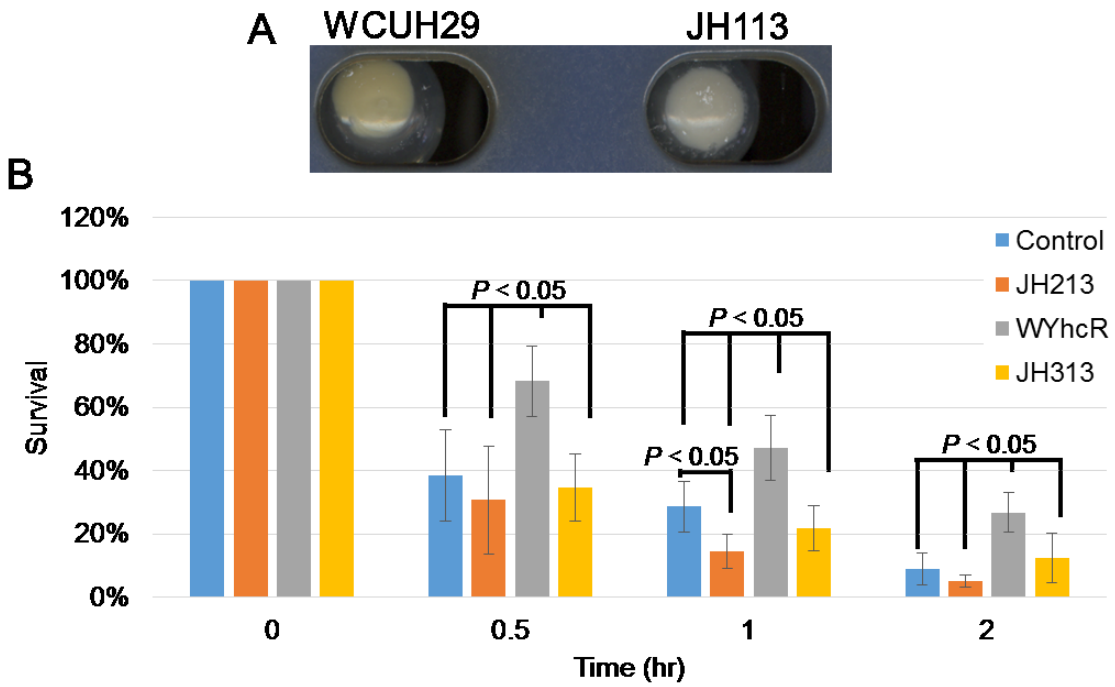

Figure 6-5 Lack of staphyloxanthin eliminates the enhanced of *S. aureus* survival resulting from YhcR overproduction. (A) comparison of STX carotenoid color production from 24 hour aerobic cultures of *S. aureus* WCUH29 wild-type and WCUH29Δ*crtM* (JH113). **(B)** Percent survival of the *S. aureus* WCUH29 control, WYhcR, JH213, and JH313 strains in human blood during ATc induction. The data represents the mean and standard deviation of four experiments. Statistical analysis was conducted with a Student's T-Test, $P \leq 0.05$. Cultures of *S. aureus* strains were cultured overnight with the inducer ATc (250 ng/ml) and the following day diluted and inoculated into 500 ml of fresh blood with erythromycin and the inducer ATc (250 ng/ml) and incubated at 37°C.

6.5 Discussion

The data presented in this chapter indicated the YhcSR TCS of *S. aureus* modulates the production of STX and subsequently, sensitivity or resistance to hydrogen peroxide. This modulation is likely to occur at the transcriptional level as YhcR binds the *crt* promoter as determined by a gel-shift assay. Construction of a P*crt-luxABCDE* promoter-reporter and/or qPCR is needed to verify transcriptional regulation during depletion or overproduction of Yhc(S)R.

The YhcS sensor kinase uses a [2Fe-2S] containing GAF domain to sense oxygen and phosphorylates YhcR under normal atmospheric oxygen concentrations (129). In the presence of oxygen or mild oxidants, YhcS likely phosphorylates YhcR and promotes production of STX as an antioxidant protection measure. The observation of the lack of pigment production during anaerobic growth on TSA plates is supportive of this, for in the absence of any oxidants, YhcS would be fully reduced (kinase inactive) and YhcR is not phosphorylated (129), therefore YhcR does would not promote P*crt* transcription.

The YhcS kinase function is inactivated by strong oxidation, such as in the presence of millimolar concentrations of H_2O_2 *in vitro* (129), but modeling of the neutrophil phagosome suggests H_2O_2 concentration is only in the low micromolar range (320). It is therefore suggested, mild oxidation of the YhcS [2Fe-2S] cluster may occur, even under anaerobic conditions such as those that may perhaps occur in the phagosomes of neutrophils within an anaerobic wound site (302). Oxidation of YhcS may up-regulate *crt* transcription and promote STX biosynthesis as way to counter-act oxidative killing by the neutrophil. If oxygen is

present in the phagosome, it too, would promote YhcSR mediated up-regulation of STX and antioxidant protection. In support of this suggestion, STX has been shown to be important for bacterial survival when *S. aureus* is cultured with neutrophils or in human and mouse whole blood. Furthermore, genetic mutations or pharmacological inhibition of the NADPH oxidase pathway in neutrophils eliminated the necessity for STX by *S. aureus* in both of these assays (312). Likewise, the data presented here showed that the genetic disruption of STX biosynthesis completely eliminated the enhanced survival of *S. aureus* due to YhcR-mediated STX overproduction. Investigations of this possibility are ongoing, and if supported, would implicate the YhcSR TCS as an important virulence factor for modulating expression of antioxidants and survival within phagosomes.

Chapter 7 : Thesis conclusions and future work

7.1 Conclusions

The overarching goal of this thesis research was to characterize and understand novel *cis*- and *trans*-acting factors that affect gene expression in *S. aureus*. To that end, the data presented in this thesis is the result of studies measuring the effect of *cis*-acting single nucleotide polymorphisms in a promoter and their influence on gene transcription and gene product expression. Furthermore, characterization of a novel *trans*-acting essential two-component system revealed the TCS transcriptionally regulates genes involved in cellular metabolism, homeostasis, and well-known virulence factors.

In chapter two studies, the α-toxin hyperproducing phenotype of the *S. aureus* RF122 was investigated and single nucleotide polymorphisms in the promoter region of the *hla* were identified that promote increased transcription of the gene and this was linked to the positive regulator, SarZ. Furthermore, RF122 has increased and decreased transcriptional expression of *hla* positive regulators and negative regulators, respectively, which appear to synergize and dramatically increase the expression of α-toxin. RF122 is of the predominant bovine mastitis ET3 clone. Alpha-toxin is an important for virulence factor in mastitis pathogenesis and the genetic and transcriptional changes that occur in RF122 that produce the α-toxin hyper-production phenotype likely contribute to its success as a mastitis pathogen.

The first goal of the studies on the *yhcSR* TCS was to confirm the essentiality of the TCS in the clinically relevant WCUH29 strain because,

although useful, the RN4220 laboratory strain is genetically mutated and, as a result, findings must be confirmed in a relevant clinical strain. Data presented in chapter three studies support the hypothesis that the *yhcSR* TCS is essential for *S. aureus* aerobic and anaerobic viability and that it is likely essential in other strains of clinical relevant *S. aureus*. In an effort to characterize and begin to identify the underlying mechanism for YhcSR essentiality the cellular morphology of inducible mutants was observed. YhcSR depletion resulted in extremely large bacterial cells that appear as if they're incapable of dividing properly, while the overproduction of YhcR produced uniformly enlarged cells, which may have thickened cell walls. Future analysis is needed to confirm the recent report linking YhcSR to cell-wall synthesis (137) and identify additional cell-wall synthesis genes regulated by YhcSR and investigate their relationship with YhcSR's essentiality.

Chapter four presents data on the positive transcriptional regulation of the *lacABCDE* and *opuCABC* operons by the YhcSR TCS. YhcR directly and specifically binds the promoter region of each operon and the depletion of *yhcSR* down regulates transcription from the respective promoters. The partial complementation of growth by expression of *lacABC in trans* shuttles intermediates into glycolysis suggesting the growth defect from *yhcSR* depletion is due to loss of expression of upstream glycolytic enzymes or the bacterial cell is starved for ATP, as the *lac* operon yields glyceraldehyde 3-phosphate which then can be catabolized for substrate level phosphorylation in glycolysis. While the exact link between the cellular osmotic regulatory *opuC* operon and the oxygen

sensing YhcSR TCS remains unclear, it is perhaps possible that YhcSR up-regulates *opuC* during aerobic logarithmic growth when bacterial growth is rapid and proper turgor pressure is important to maintain sustained cellular division, particularly in salt rich environments.

In an effort determine if a relationship existed between YhcSR and pathogenesis, the inducible mutants were assayed for survival in human whole blood. It was of little surprise to find the *yhcS* antisense mutant did not survive well in the blood and was killed faster than wild-type control or non-induced strains. The interesting finding was that overproduction of YhcR promoted survival of *S. aureus*. As outlined in chapters five and six, the survival of an YhcR overproduction strain is linked to the increased expression of exported proteases and biosynthesis of staphyloxanthin, respectively. YhcSR directly regulates each operon and promotes transcription of the respective operon. The overproduction of YhcR bypasses any sensory input from YhcS and promotes almost continuous transcription of YhcR regulated genes. The relationship between YhcSR, anti-phagocytosis, and survival within neutrophils needs to be further investigated as both virulence operons found so far to be regulated by YhcSR converge on this part of the innate immune system and suggest YhcSR has a prominent and important role for *S. aureus* in evading and escaping phagocytosis.

7.2 Future work

7.2.1 Analysis of *Staphylococcus aureus* NCTC8235 *yhcSR::erm* mutant

Of great interest and importance is the recent report of an antibiotic gene replacement of *yhcSR* in *S. aureus* NCTC8235 (321, 322). As discussed in chapter 3, the strain harbors a genetic mutation that inactivates the *rsbU* genes and does not have a functional alternative ς^B, thus many transcription factors, stress induced genes, and virulence factors are not expressed or their expression is dramatically altered. In an effort to understand how the *rsbU* mutation may affect the essentiality of *yhcSR*, in a suppressive manner, phage transduction or gene replacement with the original plasmid should be undertaken in an *rsbU*$^+$ strain, such as WCUH29 or even the *rsbU*$^+$ restored, NCTC8235 derivative, SH1000. Additionally, a mutation in *rsbU* should be made in WCUH29 followed by the attempt to delete the native *yhcSR* locus. If a WCUH29Δ*rsbU*Δ*yhcSR* is successfully created, a large-scale comparative transcriptional analysis, likely RNA-seq, of the parental WCUH29, WCUH29Δ*rsbU,* WCUH29Δ*rsbU*Δ*yhcSR* strains should be conducted to identify differentially expressed genes. The *rsbU* mutation is likely to lead to the aberrant up-regulation one or more essential genes or pathways that is normally regulated by YhcSR, so initial focus should center on genes or pathways that are known to be essential. To determine which genes are responsible for the suppressor ability, identified genes should be chromosomally integrated and ectopically expressed followed by an attempt to delete the native *yhcSR* locus.

7.2.2 Whole genome ChIP-seq analysis

To comprehensively identify YhcR binding sites in the *S. aureus* genome, chromosome immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq) should be carried out. The use of ChIP-seq has been used extensively in bacteria to confirm and identify novel binding sites for transcriptional regulators (323–326). The data garnered from ChIP-seq will provide the likely DNA recognition sequence of YhcR and genes transcriptionally regulated by YhcSR. The analysis is static therefore subsequent analysis using DNA footprinting and qPCR or promoter-reporter assays can confirm the binding site sequence and transcriptional regulation. Since YhcSR is essential for aerobic and anaerobic growth, ChIP-seq should be done for both conditions and a comparative analysis between the data sets can be used to identify differential binding sites between the two conditions. At one point during my thesis research I attempted to perform this assay, but technical issues prohibited the completion of the assay. After retrospective analysis, it is suggested that a three times repeated human influenza hemagglutinin (3×HA) epitope tag be placed in-frame on the carboxy terminal of YhcR for this assay. The use of a ChIP grade antibody recognizing the 3×HA would be essential to the assay. Once YhcR binding sites and potentially regulated genes are identified, a systemic analysis can be undertaken to identify genes in involved in the essentiality of YhcSR and determine how each of these genes is involved YhcSR mediated *S. aureus* fitness and pathogenesis.

7.2.3 Analysis of the importance of YhcS iron-sulfur cluster formation during phagocytosis and resistance to oxidants

It is known that STX is important for its antioxidant capacity and helps *S. aureus* survive within phagosomes. While a direct transcriptional link and an increase in STX production was shown because of YhcR overproduction, it would be of interest to determine the role of the YhcS [2Fe-2S] cluster in up-regulation of *crtOPQMN*, and possibly other virulence factors, during phagocytosis. It is unlikely that an allelic mutation of the cysteines that coordinate the iron-sulfur cluster could be obtained, as they are essential to the kinase activity of YhcS and likely to the TCS as a whole (129), instead an inducible-dominant negative strain should be constructed by cloning the *yhcS* ORF into the TetR inducible pYH4 plasmid. Using site-directed mutagenesis four cysteine → serine mutations at the four cysteine amino acid sites of YhcS can constructed. The uninduced and induced wild-type YhcS and YhcS mutant would then be assayed for their ability to promote STX synthesis *in vitro* and assayed for the ability to survive in the HL-60 opsonophagocytic killing assay. Further detailed analysis using isolated neutrophils from wild-type and gp91$^{Phox-/-}$ (Oxidative burst mutant, X-linked GCD model) mice could be used. If the induced C→S YhcS mutant strain survived worse than the induced wild-type YhcS strain in normal mouse neutrophils, but similar in the gp91$^{Phox-/-}$ neutrophils it would indicate the iron-sulfur cluster is important for virulence factor regulation during phagocytosis and suggest the iron-sulfur cluster is sensitive to oxidants within the phagosome.

7.2.4 Preliminary screening for YhcR inhibitors

The identification of novel antibiotics to combat the pervasive multi-drug resistance in *S. aureus* is of utmost importance. Novel essential proteins and pathways are excellent candidates for the development of new antibiotics. The thought behind this thinking is: the development of resistance will be slower, as the bacteria will have never encountered the new drug, and if used properly, resistance can be maintained to a low level.

To that end, the described screening assay was used to identify two novel inhibitors of the only other essential two-component response regulator in *S. aureus,* WalR (111). Briefly, the screening assay is a single plasmid based assay that contains an enhanced green fluorescent protein (*egfp*) reporter fused downstream of the *iclR* promoter. On a separate area of the plasmids, YhcR is cloned downstream of the DNA-binding domain of IclR, creating a chimeric protein. In the absence of the YhcR-IclR chimeric protein, the eGFP protein is expressed and the *E. coli* cells fluoresce. As the mode of transcriptional regulation for helix-turn-helix response regulators, homodimerization of the chimeric YhcR-IclR protein is facilitated by the dimerization domain of YhcR, which brings together the DNA-binding domain of IclR, resulting in the YhcR-IclR chimeric protein dependent repression of eGFP production. The introduction of compounds that inhibit the YhcR dimerization event will cause the *E. coli* cells to fluoresce relative to the no compound control (no fluorescence). Our laboratory currently has access to a library of 5,000 compounds, with the ability to expand the library through collaborations based at the University of Minnesota.

The work presented in this dissertation identified *cis*-acting SNPs and a *trans*-acting transcription factor that promote an α-toxin hyperproduction phenotype that is likely involved in increased bovine mastitis pathogenesis of *S. aureus* strain RF122. Additionally, the essentiality of the *trans*-acting YhcSR TCS was confirmed. The YhcSR TCS was identified to transcriptionally activate four operons that are involved in lactose catabolism (*lacABCD*), cellular osmotic homeostasis (*opuCABC*), and virulence factor production (*sspABC* and *crtOPQMN*). These operons contribute to *in vitro S. aureus* viability and survival of *S. aureus* in human blood.

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Appendices

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